PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/49, 15/62, C07K 14/16, 19/00, 14/03, A61K 39/21, 39/245

A2

(11) International Publication Number: WO 96/30523

(43) International Publication Date: 3 October 1996 (03.10.96)

(21) International Application Number: PCT/EP96/01433

(22) International Filing Date: 1 April 1996 (01.04.96)

(30) Priority Data:
95 10 4848.7 31 March 1995 (31.03.95) EP
(34) Countries for which the regional or international application was filed: DE et al.
95 10 4849.5 31 March 1995 (31.03.95) EP
(34) Countries for which the regional or international application was filed: DE et al.
95 10 4850.3 31 March 1995 (31.03.95) EP
(34) Countries for which the regional or international application was filed: DE et al.

(71)(72) Applicant and Inventor: WOLF, Hans [DE/DE]; Josef-Jägerhuber-Strasse 9, D-82319 Starnberg (DE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: WAGNER, Ralf [-/DE]; Franz-von-Taxis-Ring 4, D-93049 Regensburg (DE). DEML, Ludwig [-/DE]; Schneitweger Strasse 30, D-93128 Regensburg (DE). OSTERRIEDER, Klaus [-/DE]; Holzhauser Strasse 30, D-86919 Utting (DE). NOTKA, Frank [-/DE]; Gebhardstrasse 5, D-93059 Regensburg (DE).

(74) Agent: VOSSIUS & PARTNER; P.O. Box 86 07 67, D-81634 München (DE).

(81) Designated States: BR, CA, CN, JP, US, ARIPO patent (KE, LS, MW, SD, SZ, UG), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: ANTIGEN PRESENTATION SYSTEM BASED ON RETROVIRUS-LIKE PARTICLES

(57) Abstract

The present invention relates to the provision of an antigen presentation system on the basis of a DNA sequence encoding a protein capable of self assembly into particles without a lipid membrane, preferably a retroviral group-specific antigen (gag) that can be used, e.g., for the preventive and therapeutic immunization of mammals against infectious diseases or neoplasias.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgystan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic			SE	Sweden
CG	Congo	KR	Republic of Korea	SG	Singapore
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LR	Liberia	SZ	Swaziland
CS	Czechoslovakia	LT	Lithuania	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	LV	Latvia	TJ	Tajikistan
DK	Denmark	MC	Monaco	TT	Trinidad and Tobago
EE	Estonia	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	UG	Uganda
FI	Finland	ML	Mali	US	United States of America
FR	France	MN	Mongolia	UZ	Uzbekistan
GA	Gabon	MR	Mauritania	VN	Viet Nam

ANTIGEN PRESENTATION SYSTEM BASED ON RETROVIRUS-LIKE PARTICLES

Technical field of the invention

The technical problem underlying the present invention is to provide an antigen presentation system on the basis of retroviral group specific antigens (*gag*) that can be used for the preventive and therapeutic immunization of mammals against infectious diseases or neoplasias. This invention particularly relates to a newly designed antigen delivery system produced in appropriate expression systems e.g. recombinant baculoviruses, semliki forest viruses or stably transfected insect or mammalian cells, respectively. The invention is based on retroviral group specific antigens such as the human immunodeficiency virus type 1 (HIV-1) Pr55^{gag} precursor protein constituing immature forms of retroviral particles when expressed in eucaryotic cells. The immunogenicity of the immature virus-like particles (VLPs) can be extended by anchoring complete proteins on the surface of the VLPs. These proteins can either constitute autologous membrane proteins derived from the same retrovirus as the particulate *gag* carrier component or from any other virus, infectious agent or neoplastic cell. Stable anchoring of the autologous or "foreign" proteins on the surface of the VLPs requires the co-expression of the retroviral *gag*-precursor together with the antigen to be presented. In addition the antigen to be presented must include an aminoterminal signal sequence as well as a transmembrane domain in order to allow transport of the antigen via endoplasmic reticulum to the cytoplasmic membrane and stable anchoring on the surface of the budding VLP. To demonstrate the universal principle of this novel antigen presentation system, we generated recombinant VLPs on the basis of the HIV-1 Pr55^{gag} precursor allowing the presentation

- of the complete HIV-1 envelope protein gp160,
- of a derivative of the HIV-1 external glycoprotein gp120 anchored on the surface of the VLPs via a heterologous transmembrane domain derived from the Epstein-Barr virus major membrane antigen gp220/350 and
- of an equine herpesvirus glycoprotein (gB) to the immune system.

In complete absence of adjuvants, these spiked and nonreplicating/noninfectious VLPs resulted in different animal models in the induction of a humoral as well as cell mediated immune response and, when tested, in a protective immunity.

Background art

Deeper insight into pathobiological processes induced by viral infections or the generation of neoplasias resulted in the development of preventive and therapeutic strategies. Many of these strategies include the assistance of the hosts immune response. An improved understanding of of the immunological network including a variety of immune competent cell types, cytokines and antigen presentation pathways now allows the induction of defined arms of the immune response on a rational basis. The induction of a cell mediated immunity seems to play a key role in controlling e.g. the human immunodeficiency virus (HIV) during the early and asymptomatic phase of HIV infection. Similar observations are true for the control of tumor growth by the patients cellular immune resopnse. One of the key issues to achieve the induction of a cell mediated in addition to a humoral immune response is the development of appropriate and - most importantly for future application in humans - safe antigen presentation systems.

The mode of processing and presentation by antigen presenting cells (APC) determines which T-cell effector functions are specifically activated in an immune response to a protein antigen. In the most simplistic view two alternative processing pathways are distinguished: In the *exogeneous processing pathway* proteins in the extracellular fluid or in the cell membrane enter the APC through the endocytic pathway to be denatured and proteolytically degraded to peptides 12-15 residues long in an acid milieu at a late endosomal stage. Peptides generated in this pathway bind to MHC class II molecules, transit to the APC surface, and selectively stimulate $CD4^+$ T cells. Immunization with soluble protein antigens thus stimulate preferentially $CD4^+$ T cells (Germain, 1991; Germain and Hendrix, 1991).

$CD8^+$ cytotoxic T lymphocytes (CTL) are selectively stimulated in the alternative endogenous processing pathway. Intracellular proteins are degraded to peptides of an optimal 8-15 residue size in the cytosol. These peptides are transported into the endoplasmic reticulum (ER) where they bind to nascent MHC class I heavy chain ß2m microglobulin dimers. This generates transport competent trimeric complexes that move rapidly by the default secretory route to the surface membrane of the APC. Peptides presented in the context of class I molecules stimulate selectively $CD8^+$ T cells (Yewdell and Bennink, 1992; Townsend and Bodmer, 1989).

An increasing unpopular way to overcome the problem of antigen delivery to the endogenous processing pathway is the use of live recombinant, viral vectors such as recombinant vaccinia or adenoviruses. However, the application of these strategies in compromised patients is hardly acceptable from a safety point of view. Instead, a number of different formats have been devised for the presentation of selected epitopes or proteins to the immune system by nonreplicating reagents including short

lipopeptides, incorporation of proteins into ISCOM particles (Takahashi *et al.* 1990; van Binnendijk *et al.* 1992; Larsson *et al.* 1993) or liposomes (Collins *et al.* 1992; Huang *et al.* 1992; Lopes and Chain, 1992; Nair *et al.* 1992; Nair *et al.* 1992; Reddy *et al.* 1992; Zhou *et al.* 1992; Chen *et al.* 1993) or associated with detergent type adjuvants like saponin (Newman *et al.* 1992) or squalene (Raychaudhuri *et al.* 1992). Particulate carrier systems which were also demonstrated to induce $CD8^+$, MHC class I-restricted cytotoxic T cells in vivo are mainly based on viral antigens or on the yeast TY-particles (Layton *et al.* 1993; Martin *et al.* 1993). These antigen presentation systems appear to evoke strong immune responses without need of additional adjuvants, but suffer from the presentation of a limited number of relevant "foreign" epitopes.

In order to develope a nonreplicating, recombinant HIV-vaccine, we investigated the possibility of constructing an antigen delivery system based on recombinant HIV-1 Pr55*gag* VLPs mimicking immature HIV virions. This approach allows the presentation of additional, selected epitopes by a highly immunogenic relevant carrier which has been demonstrated previously to contribute to beneficial immune responses by inducing inhibitory antibodies (Papsidero *et al.* 1989; Wagner *et al.* 1992) and cytolytic T-lymphocytes (Nixon *et al.* 1988; Nixon *et al.* 1990; Phillips and McMichael, 1993). The formation of noninfectious, morphologically immature HIV-1 retrovirus-like particles (VLPs) solely depends on the expression of the myristoylated HIV-1 *gag*-polyproteins (Gottlinger *et al.* 1989). Accordingly, the production of recombinant VLPs has been demonstrated by transiently (Lopes and Chain, 1992) or stably transfected eucaryotic cells (Krausslich *et al.* 1993) and after infection of different host cells with recombinant vaccinia- (Karacostas *et al.* 1993; Wagner *et al.* 1991) or baculoviruses (Gheysen *et al.* 1989; Wagner *et al.* 1992). On the basis of these recombinant HIV-1 Pr55*gag* VLPs, we constructed a novel *per se* highly immunogenic antigen presentation system which allows the presentation of selected, immunologically relevant epitopes to the immune system.

This concept follows the construction of Pr55*gag* expression cassetts allowing the insertion of carefully selected epitopes from HIV reading frames other than *gag*. Resulting chimeric proteins should assemble into premature VLP when expressed in eucaryotic cells and allow the presentation of additional imunologically relevant epitopes. This concept also allowes to exclude epitopes suggested to be associated with adverse side effects such as induction of graft versus host-like diseases, antibodies enhancing the infection of $CD4^+$ cells by HIV or gp120 mediated apoptosis. Carefull deletion analysis within Pr55*gag* revealed two domains located within p24CA (aa 211-241) and within the p6LI moiety (aa 436-471), which are dispensable for the assembly of the mutants to premature VLP. Consequently either (i) the gp120 principal neutralizing determinant V3 or (ii) the CD4-binding domain or (iii) a highly conserved gp41 neutralizing epitope were inserted into these susceptible

sites of the Pr55*gag*-deletion constructs or fused to the carboxyterminus of the complete precursor protein. Following expression of these chimeric constructs by recombinant baculoviruses in insect cells chimeric VLP resembling immature virions could be readily rescued and purified from the cell culture supernatants in good purity and yields (Wagner *et al.* 1994).

To analyze the immunogenic potential of these antigens, different preparations of purified VLP have been administered in four week intervalls to four groups of rabbits, respectively. The immunisation with all chimeric VLPs resulted in high antibody titers of 1/100000 to the Pr55*gag* carrier component. However, the induction of insert specific antibodies and neutralisation of the homologous virus depended critically on the position of the inserted epitope within the gag-carrier polypeptide. Administration of Pr55*gag* VLPs with complete freund's adjuvant did not significantly increase the antibody titers or neutralisation potential of the resulting antisera. In comparison purified monomeric polypeptides have been by far less immunogenic as compared to the preperations of recombinant VLPs.

For many viral infections cell mediated immunity, in particular CTL response, plays a crucial role in controlling diesease. There is now ample evidence to suggest that this may also be the case in HIV-1 infection. Brand new data from "long term non progressors" indicated that a broard and complex CTL response might considerably contribute to the control of an HIV-1 infection. The findings by Takahashi and coworkers demonstrating the V3-IIIB loop to contain a H2-D^d restricted CTL epitope for BALB/c mice represents an usefull and fast accessible animal model to investigate the induction of a CTL response by rationally designed antigens (Takahashi *et al.* 1988). As demonstrated previously the immunisation of BALB/c mice with three variants of Pr55*gag*/V3 recombinant vaccinia viruses resulted in a strong $CD8^+$ CTL response, irrespective of the position of the V3-loop within Pr55*gag*. This indicated that - *by using a replicating vector* - altered flanking sequences do not negatively influence processing and presentation of the V3 peptide from the tested chimeric polypeptides (Wagner *et al.* 1993).

However, as evidenced above, it seems to be possible to induce $CD8^+$ CTL by exogenously applied lipoprotein particles, lipoproteines or liposome mediated protein transfer. Therefore we tested the capacity of chimeric Pr55*gag*/V3 VLP to induce a V3-specific $CD8^+$ CTL response. Therefore different VLP preparations were injected either intraperitoneally (IP), subcutaneously (SC) and intravenously (IV) into BALB/c mice. five days post immunization. spleen cells from primed mice were transferred into culture and restimulated with V3 peptide-labelled syngenic P815 cells in a 5 day mixed lymphocyte-tumor cell culture (MLTC). After the 5 days *in vitro* restimulation, effector cells were tested for specific cytotoxic activity. Target cells in the standard ^{51}Cr release assay were syngeneous A20 or P815 cells labelled with a 16-mer V3 consensus peptide (RIRIGPGRAFVTIGKI) previously demonstrated to be recognized

by V3 specific CTL (Wagner *et al.* 1992). The induction of V3 specific CTL strictly depended on the dose of administered antigen ranging from 20µg to 100 ng, which was still considered positive. The route of adminsttration whether IP, SC or IV did not influence the CTL reactivity. V3 specific CTL were not only found in splenocytes, but in lymphnodes when tested. Immunization of BALB/c mice with naked Pr55*gag*/V3 VLP efficiently primed the CTL response in absence of adjuvant or replicating vector (69% specific lysis): In contrast VLP adsorbed to alum or emulsified in IFA only weakly stimulated CTL response (24%, 37% specific lysis). As demonstrated above for different types of Pr55*gag*/V3 recombinant vaccinia viruses, the position of the V3-domain within different variants of chimeric VLP (Pr55*gag*/V3-3, Pr55*gag*/V3-4, Pr55*gag*/V3-5) did not influence the induction of a V3-specific CTL response. In comparison only weak priming of CTL was detected for in vivo priming with recombinant gp160. Immunisation of Pr55*gag* VLP or V3-16mer peptide was not sufficient for priming a specific CTL response. These data clearly demonstrate that recombinant chimeric VLP represent useful tools for inducing a strong, specific $CD8^+$/CTL response *in vivo* in addition to a humoral immunity.

Recently the induction of antibodies has been proven for HIV patients as well as for immunized chimpanzees neutralizing a variety of different HIV strains by recognizing conserved conformations within the gp120 external glycoprotein (Steimer and Haigwood, 1991). In order to be capable of inducing this antibody population

- **in addition to an efficient CTL response**
- **in complete absence of adjuvants**
- **and in absence of replicating vector**

we established a novel approach, which allows stable and covalent anchoring of gp120 or derivatives thereoff on the surface of the recombinant HIV-virus like particles by a heterologous transmembrane (TM)-region. Here, we describe the presentation of the complete external glycoprotein or chimeric derivatives thereof to the immune system. In addition we extended this antigen delivery system towards heterologous proteins derived from viruses other than HIV such as Epstein-Barr virus (EBV) or equine herpesviruses (EHV-1). In all cases tested, we were able to demonstrate the induction of a cell mediated in addition to a humoral immune response in experimental animals.

Brief summary of the invention

Thus, the technical problem underlying the invention is to provide DNA sequences encoding authentic or modified polypeptides derived from HIV, or from any other virus, infectious agent or neoplastic cell which allow the presentation of the polypeptides on the surface of noninfectious retroviral virus-like particles (VLPs).

The solution of the above technical problem is achieved by providing the embodiments characterized in the claims.

Accordingly, the present invention relates to the presentation of immunologically important epitopes, authentic or chimeric polypeptides via noninfectious retrovirus-like particles to the immune system.

In a preferred embodiment, the retrovirus-like particulate carrier is encoded by the group specific antigen (gag) of a retrovirus being pathogenic to humans, subhuman primates or other mammals.

In a particularly preferred embodiment the DNA sequence encoding the retrovirus-like particles is derived from any of the retroviruses HTLV-1, HTLV-2, HIV-1, HIV-2, SIV or FIV.

In a further particularly preferred embodiment the gag polypeptid is pr55^{gag} of HIV-1.

Depending on the host organism used, the gag polypeptides spontaneously form said retrovirus-like particles.

In another preferred embodiment the retrovirus-like particles, which are composed by retroviral gag polypeptides are spiked by additional immunologically relevant peptides or proteins which are presented to the immune system.

These immunologically relevant peptides or proteins can be derived from any infectious agent or neoplastic cell.

In a preferred embodiment, the proteins to be presented by the retrovirus-like particles represent authentic (occurring in nature) or chimeric (not occurring in nature) membrane proteins.

In a particularly preferred embodiment, these membrane antigens are derived from different viruses such as retroviruses or herpesviruses.

In a further specific embodiment the envelope proteins being anchored on the surface of a retrovirus-like particle are derived from any of the retroviruses HTLV-1, HTLV-2, HIV-1, HIV-2, SIV or FIV or the Epstein-Barr (EBV) virus or the equine herpesvirus EHV.

More specifically the antigen anchored on the surface of said retrovirus-like particles is the complete envelope protein gp160 of HIV-1 or the major membrane antigen of EBV gp220/350 or the herpes simplex virus gB homologue of EHV.

In another particularly preferred embodiment, the transmembrane and cytoplasmic domain of a given membrane protein may be replaced by a heterologous membrane anchor sequence. This heterologous transmembrane domain may be encoded by any viral envelope protein or cellular membrane protein.

The above mentioned transmembrane domain including a short cytoplasmic tail is derived from the Epstein-Barr virus major membrane antigen gp220/350.

The DNA sequence encoding the EBV gp220/350 transmembrane domain including a short cytoplasmic tail is fused by a short linker sequence encoding a flexible glycin/serin stretch to the DNA sequence encoding different derivatives of the HIV-1 external glycoprotein gp120.

More specifically, a 5´ DNA sequence derived from the Interleukin-3 (IL-3) gene and encoding the IL-3 signal peptide is connected via a short multiple cloning site with the DNA sequence encoding the glycin/serin linker and the EBV gp220/350 transmembrane anchor sequence.

The multiple cloning site allows the insertion of any other DNA sequence encoding an immunologically relevant protein.

More importantly, the NH_2 terminal fused IL-3 signal sequence induces the transport of the chimeric proteins via endoplasmic reticulum to the cytoplasmic membran upon expression of the construct in eucaryotic cells. Anchoring of said chimeric polypeptides in the cell membrane is achieved by the EBV gp220/350 transmembrane anchor sequence fused to the COOH-terminus of the chimeric polypeptides.

Depending on the host organism used, co-expression of the above mentioned retroviral gag polypeptides with authentic (occurring in nature) or chimeric (not occurring in nature) membrane proteins spontaneously leads to the formation retrovirus-like particles, which are spiked with the authentic or chimeric membrane proteins.

Depending on the host organism and culture conditions used, said spiked retrovirus-like particlesare secreted into the cell culture supernatantallowing the recovery of the expression product from the medium.

Its a further specific embodiment of the invention to produce said spiked retrovirus-like particles (i) in a baculovirus dependent expression system in insect cells, (ii) in stably transfected Drosophila Schneider cells, (iii) in a Semliki-Forest virus driven expression system or (iv) in any other mammalian cell line such as CHO cells.

Polyvalent antigens which contain at least one antigenic domain are suitable for diagnosis of a variety of infectious agents and neoplasias based on antibodies binding to the antigens presentet on the surface of retrovirus-like particles.

Said recombinant VLPs represent a pharmaceutical composition delivering at least one antigenic domain suitable for prevention and therapy of a variety of infectious agents and neoplasias to the immune system by means of inducing a humoral and cell mediated immune response.

Administration of said VLPs represents a general method of preventing or treating HIV-infection. EBV-infection or EBV-related diseases and EHV-infection after administration to humans or horses in amounts sufficient to modulate or induce an immune response.

Brief description of the figures

Figure 1: Scematic drawing illustrating the construction of the plasmids encoding the chimeric gp160 and gp120 genes gp160, gp120/TM, gp120^{5-}/TM and gp120^{20-}/TM.

The numbers below the hatched boxes refer to the 1st nucleotide of the coding region (A of ATG start codon); ⎯ refers to synthetic oligonucleotides (Ol)

(A) plin20 was generated from pUC8 by insertion of 2 annealed oligonucleotides Ol 1 and Ol 2 (MCS = multiple cloning site of 56 nucleotides). The restriction sites included within the MCS are indicated.

(B) Ol 3a and Ol 4a were synthetic oligonucleotides used to generate a PCR fragment encoding the IL-3 signal peptide from annealed and filled up oligonucleotides Ol 3 and Ol 4. The redigested 75 nucleotide (nc) PCR product was inserted into the EcoRI/KspI site of plin20 to generate plin20-S.

(C) Ol 5 and Ol 6 were used to generate a PCR fragment encoding the complete gp160 gene lacking the 30 aa NH_2-terminal signal peptide. After redigestion with KspI/PstI the resulting 2481bp PCR fragment was inserted into plin20-S to generate plin20-S-gp160

(D) Ol 7 and Ol 8 were used to generate a PCR fragment encoding a 6 aa Gly/Ser hinge strech, fused to the EBV gp220/350 transmembrane domaine NH_2-terminus. The MroI/PstI redigested PCR fragment (153 bp in length) was inserted into the MroI/PstI site of plin20-S to generate plin20-ST.

(E) Ol 5 and Ol 9 were used to generate a PCR fragment encoding the gp120 gene lacking the 30 aa NH_2-terminal signal peptide. After redigestion with KspI/MroI the resulting 1431 bp PCR fragment was inserted into the KspI/MroI site of plin20-ST to generate plin20-S-gp120-T.

Figure 2: Expression of the rgp160 and chimeric rgp120 derivatives in insect cells.

Spodoptera frugiperda cells were infected with recombinant baculoviruses rAc160 (lane 4), rAc120/TM (lane 5), rAc120^{5-}/TM (lane 6) and rAc120^{20-}/TM (lane 7) at a MOI of 10. For control. Sf 9 cells were either not infected (lane 1), or infected with wildtype baculovirus (lane 2) or a recombinant baculovirus expressing the HIV-1 Pr55^{gag} gene product (rAcgag; lane 3). Correct expression of the different HIV-1 gp160/120 derivatives was proven by analyzing extracts of 10^4 infected cells harvested 3 days p.i. by conventional Western blot analysis. Recombinant proteins

were detected by monoclonal antibodies directed to the third variable domain V3 of gp120 (A) (DuPont, NEA 9303) and the HIV-1 transmembraneprotein gp14 (B) (DuPont, NEA 9305). Positions of the molecular weight standart are given from the left, positions of specifically detected recombinant proteins are indicated at the right side of the figure.

Figure 3: Co-expression of the chimeric HIV-1 envelope proteins in insect cells.

For coexpressing HIV-1 Pr55^{gag} with different variants of the chimeric HIV-1 envelope proteins, HighFive insect cells were co-infected with a Pr55^{gag} recombinant baculovirus (rAcgag) and a recombinant baculovirus expressing one of the HIV-1 envelope constructs rAc160 (lane 4) or rAc120/TM (lane 5) or rAc120^{5}-/TM (lane 6) or rAc120^{20}-/TM (lane 7) at a MOI of 10 for each virus, respectively. Co-expression of both components was demonstrated in cell lysates of co-infected insect cells by conventional Western blot analysis as described above by using a monoclonal antibody to the HIV-1 p24 capsid moiety within the Pr55^{gag} precursor (16/4/2) (A) and to the gp120 V3-domain (B). Positions of the molecular weight standart are given from the left, positions of specifically detected recombinant proteins are indicated at the right side of the figure.

Figure 4: Expression of gp160 or derivatives thereoff on the surface of recombinant retrovirus-like particles (VLP).

A, B: Serum free cell culture supernatants were harvested four days after co-infection of 10^6 HighFive insect cells with rAcgag and rAc160 (lane 4) or rAc120/TM (lane 5) or rAc120/^{5}-TM (lane 6) or rAc120/^{20}-TM (lane 7) at a MOI= 10 for each virus. For control, Sf 9 cells were either not infected (lane 1), or infected with wildtype baculovirus (lane 2) or coinfected with wildtype baculovirus and a recombinant baculovirus expressing the HIV-1 Pr55^{gag} gene product (rAcgag; lane 3). The supernatants were separated by isopycnic sucrose sedimentation analysis. 600 µl aliquots were analyzed by using a commercial p24 sandwich assay (Abbott). Characterization of the antigenic peak fraction by immunoblotting using monoclonal antibodies to p24 (16/4/2) (A) and to the V3-domain within gp120 (B) revealed coincidence of the Pr55^{gag} precursor and the envelope protein derivatives in the antigenic peak fraction. C, D: Immunoprecipitations from the antigenic peak fractions were performed with 10 µl of a gp120 V3-loop specific murine monoclonal antibody (DuPont, NEA 9303). Immunoprecipitates were separated by SDS-Page and analyzed after conventional western blotting. Recombinant antigens were detected by using using monoclonal antibodies to p24 (16/4/2) (C) and to the V3-domain within gp120 (D). Positions of the molecular weight standart are given from the left,

positions of specifically detected recombinant proteins are indicated at the right side of the figure.

Figure 5:

Recombinant Pr55^{gag}/env VLP, but not HIV-1 V3-loop derived peptides primed V3-specific CTL from BALB/c mice. BALB/cJ mice (H-2^{d}) were either not primed or were primed *in vivo* by a single injection of either 6µg of Pr55^{gag} VLP or chimeric Pr55^{gag}/env VLP or 50µg of a 16mer V3-peptide, in absence of adjuvants. The *in vitro* restimulation of CTL and cytotoxity assay was carried out as described in the examples.

Figure 6:

Virus-like particles (VLPs) spiked with gp14 were generated in insect cells by co-infection of two different recombinant baculoviruses. 4 days p.i. supernatants were harvested and the particles were collected by isopycnic centrifugation in a sucrose gradient and checked for purity by electron microscopy. Five microliters of these preparations were run in a 15% SDS-PAGE, transferred to nitrocellulose and probed with anti-HIV gag mab 16/4/2 or with anti-EHV-1 serum 528/84. HIV-VLPs produced by infection with rAcgag alone and harvested at 72 h p.i. were used as a control (lane Co). The MWs of the reactive proteins are indicated in kD.

Figure 7: Immunoelectron microscopy of VLPs.

VLP-gp14 preparations were adsorbed to grids and incubated with anti-gp14 mab 3F6. Bound mab 3F6 was detected with an anti-mouse IgG gold conjugate and and analyzed by electron microscopy. A representative immunogold labeled particle is shown.

Figure 8: DTH response in immunized BALB/c mice

DTH response in mice immunized with different gp14-preparations. Panel A shows the mean increases in ear thickness of two individual mice after i.m. immunization at 0, 24 and 48 h post inoculation of inactivated RacL11 and uninfected cell culture supernatants (see Materials and Methods). Panel B shows the DTH response of mice immunized i.nas. with the same antigens. Standard deviations ranged from 0 to 4% and are not shown.

Figure 9:

Mean weight losses of immunized animals after challenge infection with wt EHV-1 at different days after challenge infection. Panel A shows the development of mean weights after i.m. immunization and subsequent challenge, panel B shows mean weights after i.nas. immunization. There were no to moderate losses in mean body weights in all groups except for mice immunized i.nas. with pDES1, where a marked reduction of mean body weights was observed after EHV-1 challenge infection. Values are given in percent of the scores obtained at day 0 (pre-challenge). Standard deviations ranged from 0 to 5.2% and are not shown.

Figure 10:

Mean virus titers of left lung lobes of two individual mice collected on days 1, 3, 5, and 8 post challenge after i.m. (Panel A) or i.nas. immunization (Panel B). Standard deviations are shown as bars. The limit of detection was 10^1 PFU per organ and the cases where no virus from tissues was recovered is indicated by <1. Stars indicate that the difference of the respective mean values was significant ($p<0.05$) by analysis of variance and subsequent Bonferroni comparisons to the values obtained for BSA-immunized mice.

Examples illustrating the invention

Example 1: Development of vector modules allowing the construction of authentic or chimeric membrane proteins (fig. 1).

In order to be capable of inducing HIV-spezific neutralizing antibodies in addition to a HIV-envelope specific CTL response we established a novel approach, which allows stable and covalent anchoring of gp120 or derivatives thereoff on the surface of the recombinant HIV virus-like particles by a heterologous transmembrane (TM)-region. Using this strategy well documented immunological side effects associated with the gp41 transmembrane protein can be excluded. To allow stable presentation of HIV-1 gp120 epitopes in a correct, immunologically relevant conformation, we constructed recombinant baculoviruses expressing chimeric gp120 derivatives, which are covalently linked via their COOH-termini to a heterologous type 1 transmembrane moiety (TM) of the Epstein-Barr-Virus (EBV) gp250/350. Both domains are seperated by a flexible $(gly/ser)_3$ hinge region to allow independent folding of both domains. To avoid either unspecific cleavage of the gp120 at carboxyterminal cleavage sites or uncorrect folding of the *env*-chimeras, we additionally established derivatives truncated from the COOH-terminus by either five (gp120/5$^-$) or 20 amino acids (gp120/20$^-$), linked to the TM. In a second set of DNA constructs, the original gp160/120 signal peptide encoding sequence was replaced by a DNA sequence encoding the signal peptide of interleukin-3 (IL-3). The construction of the chimeric envelope proteins was performed as follows in detaill:

(A) Construction of plin20: In order to establish a vector system for convenient cloning of authentic or chimeric membrane proteins, we replaced the EcoRI/HindIII multiple cloning site (MCS) by a new MCS including the restriction sites 5´-EcoRI-KspI-SacI-BglII-XbaI-SalI-XhoI-MroI-PstI-3´ by ligating two annealed complementary oligonucleotides into a EcoRI/HindIII linearized pUC 8. The synthetic oligonucleotides 1 and 2 (refered to as OI 1 and OI 2 respectively) and all additional oligonucleotides mentioned in the following text are given in the appendix. The HindIII restriction site was disturbed by the cloning procedure.

The following constructions were all accomplished by polymerase chain reaction (PCR) procedures under standard PCR conditions. Besides amplifying the desired nucleotide sequences, restriction sites flanking the coding regions were introduced for more convenient cloning procedures by means of 5´-overhanging primers. The resulting reaction products were verified after subcloning by double stranded DNA sequenzing utilizing a 373A DNA Sequencer (Applied Biosystems).

(B) Construction of plin20-S: The 5´ 75 nucleotides of the murine interleukin 3 (IL-3) gene encoding a eucaryotic signal sequence were cloned into the above described plin20 vector. Two overlapping synthetic oligonucleotides (OI 3 and OI 4) served after

annealing and filling up the protruding single stranded DNA sequences as template in a PCR (reaction 1). Two amplification primers OI 3a and OI 4a were used to amplify the IL-3 leader template and to introduce terminal restriction sites. The reaction yielded a double-stranded synthetic oligonucleotide containing a EcoRI-restriction site at the 5´- and a KspI-restriction site at the 3´-end. To generate the plin20-S plasmid this double-stranded oligonucleotide was cleaved with EcoRI/KspI and inserted into the EcoRI/KspI linearized plin20.

(C) Construction of plin20-S-gp160: To construct a full-length HIV-1 gp160 gene, having the natural signal sequence replaced by the IL-3 signal-peptide, the plasmid pNL4-3 containing the entire HIV-1 genome was used as the source for amplification of the HIV-1 envelope glycoprotein (gp) coding sequence. Using the oligonucleotides 5 and 6 in a PCR reaction, a 1.4 kb fragment containing a KspI restriction site at the 5´- and a PstI restriction site at the 3´-end was amplified and subsequently cloned into a KspI/PstI digested plin20-S vector. The introduction of the 5´Ksp restriction site into the gp 160 open reading frame resulted in a conversion of the residues 32 (E) and 33 (K) of HIV-1 HX-10 isolate to A, E, N. The subcloned HIV-1 gp160 fragment reasembles the gp160 sequence from amino acid (aa) position 31 to 856 (nucleotide position 6314-8791).

(D) Construction of plin20-ST: To generate the plin20-ST plasmid the coding region of the EBV gp 220/350 transmembrane (TM) domaine was amplified in a PCR reaction using the plasmid pBRBamHI-L as a template and the oligonucleotides 7 and 8 as primer. Furthermore the oligonucleotides 7 and 8 introduced a MroI restriction site at the 5´- and a PstI restriction site at the 3´-end respectively, flanking the EBV gp220/350 TM coding sequence. The 5´primer OI 7 additionally accomplished the fusion of the Gly/Ser hinge region coding to the 5´ end of the EBV gp220/350 TM domaine coding nucleotide-sequence. The PCR product was digested with MroI/PstI and ligated into the plin20-S vector. The position of the cloned EBV fragment in the virus genome is nucleotide 89433-89576 on the complementary strand corresponding to aa 860 - aa 907 of the EBV gp220/350 (EBV B95-8, Baer et al. 1984, Nature 310: 207-211; Genebank, accession V01555).

(E) Construction of plin20-S-gp120 (variants) -T: For the introduction of the different HIV-1 gp120 derivatives (120/TM, 120^{5-}/TM and 120^{20-}/TM) into the plin20-TS plasmid the same source for HIV-1 coding sequences mentiond under (C) was used. Using the oligonucleotides 5 (5´primer) and 9a or 9b or 9c (3´primer) in a PCR, DNA fragments encoding truncated HIV-1 gp120 derivatives were produced (120/TM with OI 9a, 120^{5-}/TM with OI 9b and 120^{20-}/TM with OI 9c). All subgenomic fragments included a KspI restriction site at the 5´- and a MroI restriction site at the 3`-end. Using these restriction sites all three gp120 derivatives were subcloned into the plin20-ST vector. The cloned HIV-1 gp120 variants reasemble the aa sequences from residue 31 to residue 506, 502 and 487 (the corresponding nucleotide positions

are 6314 - 7741, 7729 and 7684) for 120, 120^{5}-/TM and 120^{20}-/TM respectively. In the following text, above described gp160/120 derivatives are referred to as gp160, gp120/TM, gp120^{5}-/TM and gp120^{20}-/TM.

In an analogous procedure the HIV-1 gp160/120TM derivative constructs exhibiting the autologous HIV-1 signal sequence were obtained. The PCR amplifications of above mentioned gene segments were performed using the pNL4-3 template, one 5´primer (OI 10) for all four constructs (introducing a 5´ EcoRI restriction site) and the above described 3´primer OI 6, OI 9a, OI 9b or OI 9c. The resulting DNA fragments were subcloned into plin20 derivatives. The gp160 original coding region (aa 1-856) was inserted into plin20 after EcoRI/PstI digestion and ligation. The different gp120 coding sequences (gp120: aa 1-506, gp120^{5}-: aa 1-502, gp120^{20}-: aa 1-487) were subcloned into plin20-ST using the EcoRI/MroI restriction sites.

Oligonucleotides used:

OI 1	5´-AATTCAATCCGCGGGAGCTCAGATCTAGAGTCGACTCGAGTCCGGAAATCTGCAGT -3´
OI 2	5´-AGCTACTGCGATTTCCGGACTCGAGTCGACTCTAGATCTGAGCTCCCGCGGATTG -3´
OI 3	5´-ATATTAGAATTCGCCATGCTATTACTACTTCTTATGCTATTCCATCTAGGACTACAAGCT -3´
OI 4	5´-CCTTCGCTGCAGTTCGTTCCCCGCGGTCATGTTTATGGGGTCTCGTCCTGATATTGAAG CTTGTAGTCCTAGATG -3´
OI 3a	5´-ATATTAGAATTCGCCATGC -3´
OI 4a	5´-ATACCTTCGCTGCAGTTCGTTCC-3´
OI 5	5´-ATATTAGAATTCTCGAGCCGCGGAAAACTTGTGGGTCACAGTC-3´
OI 6	5´-ATATTACTGCAGTTATAGCAAAATCCTTTC C-3´
OI 7	5´-ATATTATCCGGAAGCGGGGCAGGATCCATGCTAGTACTTCAATGGGCCTCTCTG-3´
OI 8	5´-ATATTACTGCAGTTATACATAGGTCTCGGCCTC -3´
OI 9a	5´-ATATTATCCGGACACCACTCTTCTCTTTGC -3´
OI 9b	5´-ATATTATCCGGACTTTGCCTTGGTGGGTGCTACTCC -3´
OI 9c	5´-ATATTATCCGGATTTATATTTATATAATTCACTTCTCC-3´
OI 10	5´-ATATTAGAATTCATGAGAGTGAAGGAGAAATATCAGC-3´

Example 2: Subcloning of the chimeric HIV-1 envelope genes into the baculovirus transfervector pVL1393 and construction of recombinant baculoviruses.

In order to be able of constructing recombinant baculoviruses expressing the chimeric HIV-1 envelope proteins in an insect cell expression system, the EcoRI/PstI DNA fragments encoding the gp160 and gp120 derivatives have been subcloned into the EcoRI/PstI site of the transvervector pVL1393. Plasmid DNAs have been purified by using a Quiagen tip 100 kit (Diagen). Recombinant baculoviruses encoding the gp160 (rAc160) and gp120 (rAc120/TM, rAc120/^{5}-TM, rAc120/^{20}-TM) derivatives were established and plaque purified according to standard procedures as described (Wagner et al. 1994)

Example 3: Expression of the chimeric HIV-1 envelope proteins in insect cells.

Spodoptera frugiperda cells were infected by recombinant baculoviruses (rAc160, rAc120/TM, rAc120/^{5}-TM, rAc120/^{20}-TM) at a MOI of 10. Correct expression of the different HIV-1 gp160/120 derivatives was proven by analyzing extracts of 10^4 infected cells by conventional Western blot analysis. Briefly, the cell lysates were diluted in sample buffer (Sambrook et al., 1989), separated by electrophoresis on 10-12.5% SDS-polyacrylamide gels and transferred to nitrocellulose (Schleicher and Schuell) by electroblotting. Sheets were incubated for 1 h at RT with 10% nonfat dry milk in Tris-buffered saline (TBS) containing 0.05% Tween 20 (Sigma) and washed in TBS-Tween 20. Filters were then incubated overnight at 4°C with mabs to the third variable domain V3 of gp120 (DuPont 9303). After removing the antibodies, blots were washed twice with TBS-Tween20 and incubated for 1 h at RT with anti-mouse IgG-POD conjugate. Blots were washed again and substrate (4-chloro-1-naphtole) was added (fig. 2). Exposition of the envelope derivatives on the cell surface was proven by immunoflourescence analysis and confirmed by FACSscan analysis (tab.1).

For that purpose infected insect cells were fixed with paraformaldehyde (1% in PBS). Cells were incubated with a mab directed to the V3 domain of gp120 (1/100 dilution in PBS), washed twice and incubated for 10 min at RT with an anti-mouse IgG fluoroisothio-cyanate (FITC) conjugate. After two washes in PBS, DNA was stained with propidium iodide and samples were analyzed with a fluorocytometer (FACSscan, Becton- Dickinson) or by UV-microscopy.

Example 4: Co-expression of the chimeric HIV-1 envelope proteins in insect cells.

For coexpressing HIV-1 Pr55*gag* with different variants of the chimeric HIV-1 envelope proteins, high five insect cells were co-infected with a Pr55*gag* recombinant baculovirus (rAcgag) and a recombinant baculovirus expressing one of the HIV-1 envelope constructs depicted in fig. 1 (MOI=10 for each virus). Co-expression of both components was demonstrated in cell lysates of co-infected insect cells by conventional Western blot analysis as described above by using a monoclonal antibody to the HIV-1 p24 capsid moiety within the Pr55*gag* precursor and to the gp120 V3 domain (fig. 3). FACSscan analysis of the co-infected cells by using the V3-loop specific murine monoclonal antibody demonstrated clearly that the chimeric gp120 derivatives including the COOH-terminal transmembrane domain of the EBV gp220/350 glycoprotein were incorporated into the cell membrane in 2-3 fold amounts if compared with the gp160 wildtype polypeptide (tab.1).

Example 5: Analysis of particle formation.

Ultrathin sections of insect cells coexpressing Pr55*gag* and the gp120 derivatives (fig. 1: gp160, gp120/TM, gp120/^{5}-TM, gp120/^{20}-TM) after co-infection with the respective recombinant baculoviruses revealed efficient budding of recombinant VLP (not shown). To further assess the nature of these VLPs serum free cell culture supernatants were harvested four days after the co-infection of 10^6 high five insect cells (rAcgag and rAc160 or rAc120/TM or rAc120/^{5}-TM or rAc120/^{20}-TM) and analyzed by sucrose sedimentation analysis in a gradient from 10% to 60%. Quantification of p24 antigen in different fractions by a commercial sandwich assay (Abbott) revealed retrovirus-like particles sedimenting at a density of 1.15-1.17 g/cm^3 for all co-infections tested, which is identical to the density which has been demonstrated previously for mature HIV-virions. Analysis of the antigenic peak fraction by immunoblotting using monoclonal antibodies to p24 (16/4/2) and to the V3 domain within gp120 revealed coincidence of the Pr55*gag* precursor and the envelope protein derivatives in the antigenic peak fraction (fig. 4 A, B).

Example 6: Expression of gp160 or derivatives thereoff on the surface of recombinant retrovirus-like particles (VLP).

Expression of gp160 or derivatives thereoff on the surface of recombinant retrovirus-like particles was assessed by a co-immunoprecipitation analysis from the antigenic peak fractions of the sucrose gradients. Immunoprecipitations were performed according to standart procedures (Sambrook et al., 1989) with 10 µl of a gp120 V3-loop specific murine monoclonal antibody (DuPont 9303) in absence of detergent. Immunoprecipitates were diluted in sample buffer (Sambrook et al., 1989), separated by electrophoresis on 10-12,5% SDS-polyacrylamide gels and transferred to nitrocellulose (Schleicher and Schuell) by electroblotting. Sheets were incubated for 1 h at RT with 10% nonfat dry milk in Tris-buffered saline (TBS) containing 0.05% Tween20 (Sigma) and washed in TBS-Tween20. Filters were then incubated overnight at 4°C with mabs to p24 (16/4/2) or gp120 (fig. 4 C, D). After removing the antibodies, blots were washed twice with TBS-Tween20 and incubated for 1 h at RT with anti-mouse IgG-POD conjugate. Blots were washed again and substrate (4-chloro-1-naphtole) was added. As indicated in fig. 4 C and D, the Pr55*gag* precorsor has been co-immunoprecipitated only from peak fractions of the sucrose sedimentation analysis which were derived from supernatants of co-infected cells. This clearly indicates the gp160 or derivatives thereoff are exposed on the surface of the infected cells. In addition, we confirmed that co-expression of Pr55*gag* with the wild type gp160 construct significantly reduces the exposition of the envelope protein if compared to the chimeric derivatives of gp120 (fig. 4 C, D).

These results were further confirmed by immunoelectron microscopy of the VLPs. Immunoelectron microscopy of VLPs was performed essentially as described by

Czerny and Mahnel (Czerny and Mahnel, 1990). Purified VLPs were adsorbed to grids after fixation with 2% glutaraldehyde. Grids were washed in TBS, blocked with 3% gelatine in TBS for 1 hour at RT, and incubated with the V3-specific mab in TBS for 1 hour at RT. After three washes in TBS, grids were floated on an anti-mouse IgG immunogold conjugate (Sigma, particle sice 5 nm) for 1 hour at RT. After three washes (TBS) grids were contrasted with phosphoric tungstic acid and examined in an electron microscope (Zeiss EM 10C/CR).

Example 7: Purification of recombinant VLPs

VLPs were generated by co-infection of HighFive cells with a multiplicity of infection (MOI) of 1 per cell with the recombinant baculoviruses expressing gp160 (rAc160), gp120 or dervatives thereoff (rAc120/TM, rAc120/^{5}-TM, rAc120/^{20}-TM) and rAcgag, the latter encoding the HIV 55 kD gag protein Pr55*gag*. Supernatants of infected HighFive cells were collected 4 days p.i., purified by isopycnic sucrose gradient centrifugation as described above, and checked for purity and absence of baculoviruses by electron microscopy. After dilution of the baculovirus-free fractions VLP were pelletted by centrifugation in a TFT 41.14 rotor in Kontron centrifuge and resuspended PBS.

Example 8: Induction of a humoral immune response by recombinant VLPs.

To asses the capability of different VLP preparations to induce an adequate humoral immune response, rabbits immunized each with 20 µg of the the chimeric particles in four week intervalls in complete absence of adjuvants (Tab. 2). Determination of ELISA antibody titers and quantification of neutralizing activity of the indicated antisera was performed as follows:

Antigen ELISA: Micro-ELISA plates (Greiner, Frickenhausen, Germany) were coated with 500 ng HIV-1 HX10 lysate, 500 ng recombinant p24, 80 ng rgp120 or 300 ng V3-peptide (36mer)/well in 50µl 0.05 M sodium carbonate puffer pH 9.5 at 4°C overnight in a wetchamber. Sera diluted 1:10 to 1:1000 in PBS with 3% FCS and 2% Tween-20 were added to the coated wells. After incubation for 2 hours at 37°C the plates were washed 5 times. Bound antibody was detected with horseradish peroxidase-conjugated anti-rabbit antibody (Dakopatts, Copenhagen, Denmark) at a dilution of 1:1000, followed by incubation with o-phenylendiamine-0.01% hydrogen peroxide in phosphate buffered saline (pH 6.0). The reaction was stopped by adding 1M H_2SO_4 and read at 492 nm. Values above the mean optical density +3 SD of negative controls were considered positive. All rabbits immunized with recombinant VLPs developed high titers of antibodies ranging from 1/64000 to the Pr55*gag* carrier

component to 1/64000-1/32000 towards purified gp120. Only low titers (1/256) to a 36 amino acid peptide representing the homologous V3-peptide hav been detected.

HIV-1 neutralisation assays: Serial twofold dilutions of heat inactivated serum were incubated for 1.5 h at 37°C with 50 $TCID_{50}$ of HIV-1 HX10-strain produced on MT4 cells. The virus-serum mixture was incubated for 1h at 37°C with $5x10^4$ MT4 suspension cells. After virus adsorbtion, the unbound virus was removed and 100 µl of medium (RPMI-1640 supplemented with 10% FCS) was added to each well. 7 days p.i., 100 µl of supernatant medium was removed in order to quantify the amount of virions released from the cells using a commercial p24 sandwich assay (Abbott Laboratories, Chicago Ill.). Neutralisation titres were calculated by comparison with control wells of virus only and are expressed as the reciprocal of the highest serum dilution that inhibited p24 production and release by more than 90 %. Sera from rabbits obtained after 3 immunisations were used for the neutralisation assay. As shown in table 2 all of the sera showed neutralization titers in a range of 1:128 to 1:256, depending to the Pr55*gag*/env preperation used for immunisation.

Example 9: Induction of a cell mediated immune response by recombinant VLPs

The findings by Takahashi and coworkers demonstrating the V3-IIIB loop to contain a $H2\text{-}D^d$ restricted CTL epitope for BALB/c mice represents an usefull and fast accessible animal model to investigate the induction of a CTL response by rationally designed antigens (Takahashi *et al.* 1988). As demonstrated previously the immunisation of BALB/c mice with three variants of Pr55*gag*/V3 recombinant vaccinia viruses resulted in a strong $CD8^+$ CTL response, irrespective of the position of the V3-loop within Pr55*gag* (Wagner *et al.* 1993).

To study the capability of the chimeric *gag/env* VLP to induce cytotoxic T-lymphocytes *in vivo*, BALB/cJ mice ($H\text{-}2^d$) were immunized with 10µg of the different *gag/env* hybrid VLP in complete absence of adjuvants or replicating vector. For control BALB/c mice were injected with 50µg of a 16mer V3-peptide (RIQRGPGRAFVTIGKI) or 10µg of Pr55*gag* VLP only. Lymphoid cells were prepared from immuniced mice 6 days post immunisation and cocultivated with syngenic V3-16mer peptide labelled syngenic P815 cells, irradiated with 20000rad. A control group included unprimed BALB/c cells stimulated *in vitro* with V3 peptide labelled P815 cells. Cytotoxic effector cell populations were harvested after 5 days of *in vitro* culture. The cytotoxic response was determined against the syngenic target cell line A20 pulsed for 1h with 10^{-8}M V3-16mer peptide. Negative controls were not pulsed target A20 cells in a standard ^{51}Cr release test. Neither the synthetic V3-peptide nor Pr55*gag* VLP were sufficient to prime an adequate V3-specific CTL response. A comparably weak CTL response could be demonstrated after administration of purified gp160 (fig.5). These data clearly indicate, that anchoring of

gp160 or derivatives thereoff on the surface of recombinant Pr55*gag* VLPs results in a favourable antigen presentation, which is capable of inducing a highly efficient CTL response to the presented membrane proteins.

Example 10: Presentation of membrane proteins derived from other viruses than HIV-1 by recombinant VLPs: The equine herpesvirus gp14 (gB)

VLPs were generated by co-infection of HighFive cells with a multiplicity of infection (MOI) of 1 per cell with the recombinant baculoviruses rAc17-11 expressing the EHV gp14 membrane protein (Osterrieder *et al.* 1994) and rAcgag, the latter encoding the HIV 55 kD gag protein Pr55*gag*. In a first series of experiments, the time point of maximal load of the VLPs with recombinant gp14 was determined. Supernatants of infected HighFive cells were collected at different times p.i., purified by isopycnic sucrose gradient centrifugation, and checked for purity and absence of baculoviruses by electron microscopy. Five-microliter aliquots of the resuspended VLPs were separated by PAGE, immunoblotted and detected with both anti-p24 mab 16/4/2 and horse serum 528/84. HIV-gag was present at nearly constant levels in the preparations from 12 h p.i., but gp14 was first detected on the VLPs at 36 h p.i. and reached a maximal load at 72 h p.i. (fig. 6). For all further studies, particles were harvested at 72 h p.i..

To further demonstrate that the gp14 not only co-purified with the VLPs but was incorporated into the particles, fluorocytometric studies were performed. Anti-gag mab16/4/2 and anti-gp14 mab 4B6 precipitated the VLPs as shown by the reciprocal reaction with biotinylated mabs 4B6 and 16/4/2. In the next experiments we addressed the question why gp14 was present on the VLPs although the transmembrane and cytoplasmic domain of the protein had been deleted. As shown previously, the recombinant gp14 could be demonstrated in the cytoplasm of rAc17-11 infected HighFive cells from 12 h p.i. and was present on the surface of insect cells from 24 h p.i. reaching a maximum at 72 h p.i. (Osterrieder *et al.* 1994). The protein remained on the surface of infected cells up to 120 h p.i. when almost all insect cells were dead as determined by trypan blue stain (data not shown). These results indicated that despite the truncation of gp14, the glycoprotein was stably present in the membrane of insect cells and that the transmembrane and cytoplasmic domain are dispensable for retaining of gp14 on the cytoplasmatic membrane in the insect cell system.

These results were further confirmed by immunoelectron microscopy (Zeiss EM 10C/CR) of the VLPs as described above for the gag/env chimeric VLPs by using an anti-gp14 mab 3F6 (fig.7).

Example 11: Humoral immune response to recombinant gp14 induced by chimeric VLPs in comparison to other antigen formulations

Different antigen preparations were compared with respect to the induction of a humoral, cell mediated and potential protective immune response. For that purpose, BALB/C mice were immunized with

10 µg	of pCEP-8-gp14 (rgp14 exressed in and purified from E.Coli; (Osterrieder *et al.* 1994))
	of rAc17-11-gp14 (rgp14 exressed in and purified from insect cells after infection with a gp14 recombinant baculovirus; (Osterrieder *et al.* 1994))
	of VLP-gp14 (recombinant HIV-1 Pr55^{gag} VLPs presenting gp14 on their surface)
	BSA
50 µg	of pDES-gp14 (DNA vaccine; pcDNA/Amp (Invitrogen) derived expression plasmid containing the EHV-1 gp14 without its transmembrane and cytoplasmic domain under the control of the CMV immediate early promotor; Osterrieder et al., in press)
$10^{6.5}$PFU	RacL11 (Mayr et al., 1968)
$10^{6.5}$PFU	RacH (Mayr et al., 1968)

The ELISA and NT antibody titres obtained for the specific gp14-formulations in immunized mice are summarized in table 3. The highest ELISA and NT antibody titres were observed after im. and inas. immunization with gp14-spiked VLPs. The ELISA antibody titres were even higher than those obtained after i.m. and i.nas. application of live EHV-1 virus (RacL11; RacH). Intramuscular immunization with purified gp14 produced with gp14 purified from the supernatants of insect cells 4 days p.i. with a gp14 recombinant baculovirus (rAc17-11) gave rise to antibody levels comparable to those of the VLPs, but the titres observed after i. nas. immunization were significantly lower. Immunization with both the gp14 expressed in *E. coli* (pCEP-8-gp14) or with the pDES1-gp14 DNA vaccine yielded weak ELISA titers and no neutralizing antibodies could be demonstrated after inas immunization with both formulations.

Example 12: DTH response to recombinant gp14 induced by chimeric VLPs in comparison to other antigen formulations

The DTH response after immunization with the various recombinants was assayed by determining the increase in ear thickness of two individual mice at different time points post inoculation (p. inoc.) of inactivated antigen into the ear pinna. At 24 h p.inoc., a readily detectable increase in ear thickness was observed in mice immunized with VLPs and the *E.Coli* derived gp14 (pCEP-8-gp14) via the i.m. and i.nas route. This reaction was decreasing at 48 h p.inoc.. A marked increase in ear thickness was also observed in mice immunized i.m. with purified gp14 produced by rAc17-11 in insect cells and with the pDES1 DNA vaccine. In contrast, a weak DTH reaction was detectable after i.nas. immunization with these two gp14 formulations. The results of the comparative DTH tests are summarized in Fig. 8.

Example 13: Clinical observations in mice immunized with gp14 chimeric VLPs in comparison to other antigen formulations after challenge with wt-EHV-1

Immunized mice were challenged inas with 106.5 PFU of EHV-1 wt strain RacL11. All mice immunized with BSA developed signs of illness such as ruffled fur, respiratory symptoms, and hunched posture associated with a dramatic loss of body weight of up to 23% by day 2 after challenge infection. Similar observations have been reported for EHV-1 strain Ab4 (Awan *et al.* 1990).

In contrast to others (Awan *et al.* 1990; Inazu *et al.* 1993), no deaths in BSA-immunized mice were observed after challenge infection and animals recovered from the weight losses by day 4/5 p.chall. but did not reach the preinfection weight until day 8 p.chall. In contrast, no or only a mild (up to 6%) decrease in the mean body weights was observed after challenge infection of mice immunized im with all recombinant gp 14 formulations and subsequent challenge infection. In mice immunized im with pDES1 DNA vaccine, however, one individual mouse exhibited ruffled fur, dyspnoea, and a body weight loss of 15% on Day 4 p.chall.. Similarly, no or mild mean body weight depression was observed in mice immunized inas with the VLP-gp14 preparation and the recombinant gp14 produced by rAc17-11. In pCEP-8-gp14- and pDES-1 immunized mice, one and two individual animals, respectively, showed signs of illness and body weight losses of up to 20% after challenge infection was seen in mice previously infected inas with the live virus strains RacL11 or RacH (fig. 9).

Example 14: Virus reisolation from mice immunized with gp14 chimeric VLPs in comparison to other antigen formulations after challenge with wt-EHV-1

After challenge infection, virus was recovered from lungs of two individual mice killed on days 1, 3, 5 and 8 p. chall., respectively. A significant (<0.05) reduction of lung

virus titers (103 PFU/lung) on all days p.chall. was observed in mice previously infected i.m. or i.nas. with live virus strains RacL11 and RacH when compared to BSA-immunized mice. From day 3 p.chall. on, virus titers were below 10^1 pfu/organ in the mouse groups immunized with live virus and differed significantly from the means of BSA immunized mice from day 1 to 8 p.chall. (Fig. 10). The most obvious and marked decrease in virus recovery from lungs after immunization with recombinant gp14 preparations was seen after i.m. and i.nas. immunization with the VLPs spiked with gp14. On day 1 p.chall., mean virus titers of groups 9 and 10 (Table 3) reached values of around 10^4 PFU/organ, a more than 100-fold reduction compared to BSA immunized mice. From day 3 p.chall., virus titers in lungs were below 10 PFU/organ, both after previous im and inas immunization. This reduction of virus load was obtained although the antigen preparation was not emulsified in Freund's adjuvant prior to im. immunization (Table 3). Moreover, the means of lung virus titers were reduced significantly ($p<0.05$) when compared to BSA-immunized mice on all days p.chall.. After immunization with purified rAc17-11-gp14 (insect cell derived gp14), a significant reduction ($p<0.05$) in virus load of mouse lungs was seen after i.m. immunization (emulsified in Freund's adjuvant) from day 3 p.chall.. After i.nas. immunization with that antigen, virus titers recovered from lungs were higher compared to those after immunization with RacL, RacH or the VLPs, but we were not able to demonstrate any virus on day 5 p.chall.. In pCEP-8-gp14 (*E.Coli* derived gp14) immunized mice, protection against challenge infection appeared to be efficient after both im. and inas. immunization. The virus titres in lungs were in general comparable to those seen in insect cell derived gp14 (rAc17-11gp14) immunized mice and were also reduced significantly after i.m. and i.nas. immunization from day 3 p.chall. ($p<0.05$). The lowest - but still significant - reductions in lung virus titres on days 3, 5, and 8 p.chall. were observed in mice immunized i.nas. and i.m. with 50 µg pDES1-DNA vaccine (Fig. 10).

In summary, mice immunized with gp14-spiked VLPs both i.m. and i.nas. were found to be best protected against subsequent EHV-1 challenge.

Table 1:

Surface Expression of Chimeric HIV-1 Envelope Proteins Determined by FACScan Analysis

cells infected with	mean	% of B-gp160	% of B-Pr55/B-gp160	% of single infection
not infected	11	4		
B-WT	11	4		
B-Pr55	7	2		
B-gp160	292	*100*		
B-gp120TM	407	139		
B-gp120/5TM	370	127		
B-gp120/20TM	367	126		
Coinfection				
B-Pr55/ B-WT	10	3	10	
B-Pr55/ B-gp160	96	33	*100*	33
B-Pr55/ B-gp120TM	281	96	292	69
B-Pr55/ B-gp120/5TM	190	65	197	51
B-Pr55/ B-gp120/20TM	166	57	172	15

R.W. 1995/RIMMH

Table 2: Antibody response induced by Pr55gag/env chimeric VLPs

Vaccine[a]	Ab-titer[b]				
	HIV-lysate	p24	gp120	V3	NT[c]
Pr55^{gag}	$3.2x10^4$	$3.2x10^4$	-	-	-
pr55^{gag}/gp160	$6.4x10^4$	$6.4x10^4$	$6.4x10^4$	<16	1:256
Pr55^{gag}/gp120TM	$3.2x10^4$	$3.2x10^4$	$3.2x10^4$	<16	1:256
Pr55^{gag}/gp120^{5}-/TM	$1.6x10^4$	$1.6x10^4$	$1.6x10^4$	<16	1:256
Pr55^{gag}/gp120^{20}-/TM	$1.6x10^4$	$3.2x10^4$	$1.6x10^4$	256	1:128

[a] Rabbits received 10 µg the chimeric VLP preparations in absence of any adjuvants

[b] Serum antibody levels were tested by ELISA three weeks after the second booster immunization and expressed as reciprocal of the dilution that gave rise to one half the maximal density at 492 nm (midpoint tire). The antibody levels were determined against various antigens such as HIV-1 lysate, rp24, rgp120 and a synthetic V3 peptide (36-mer). Titers below 1/16 were considered to be unspecific and reactions counted as negative.

[c] Reciprocal of the highest serum dilution that inhibited p24 production by more than 90% 7 days p.i. were classified as neutralizing.

Table 3: Immunization schedules for the different mouse groups and ELISA/SN titers

Mouse group	Antigen	Route of immunization	Days of immunization	Adjuvant	ELISA titer[a] (day 35)	SN titer[b] (day 35)
1	RacL11	i.m.	day 21	none	1:20,480	1:16
2	RacL11	i.nas.	day 21	none	1:5,120	1:8
3	RacH	i.m.	day 21	none	1:10,240	1:16
4	RacH	i.nas.	day 21	none	1:2,560	1:8
5	pCEP-8-gp14	i.m.	days 0/21	FA[c]	1:320	1:4
6	pCEP-8-gp14	i.nas.	days 0/21	none	1:80	<1:2
7	rAc17-11-gp14	i.m.	days 0/21	FA[c]	1:20,480	1:8
8	rAc17-11-gp14	i.nas.	days 0/21	none	1:640	1:2
9	VLP-gp14	i.m.	days 0/21	none	1:40,960	1:8
10	VLP-gp14	i.nas.	days 0/21	none	1:2560	1:4
11	pDES1-gp14	i.m.	days 0/21	none	1:80	1:2
12	pDES1-gp14	i.nas.	days 0/21	none	1:40	<1:2
13	BSA	i.m.	days 0/21	FA[c]	<1:10	<1:2
14	BSA	i.nas.	days 0/21	none	<1:10	<1:2

[a] Endpoint ELISA titers were determined with purified EHV-1 virions (1µg/ml) by twofold $\log_2$ dilutions of sera starting with a 1:10 dilution. Absorbances were read at 450 nm and scores were judged positive when exceeding counts of BSA-control sera by three standard deviations.

[b] SN titers were determined on Rk_{13} cells with 50 $TCID_{50}$ RacH and triplicate $\log_2$ dilutions of sera inactivated at 56° for 30 min. No complement was added. Titers reflect the serum dilution with complete protection in all wells.

[c] For the first immunization (day 0), antigens were emulsified in complete Freund's adjuvant, for the second immunization (day 21), in incomplete Freund's adjuvant. All preparations were diluted in PBS, RacL11 or RacH ($10^{6.5}$ PFU), 10 µg of recombinant antigens, 50 µg of pDES1, and 100 µg of BSA were used for each immunization.

References

Awan, A. R., Chong, Y. C., and Field. H. J.(1990). The pathogenesis of equine herpesvirus type 1 in the mouse: a new model for studying host responses to the infection. *J. Gen. Virol.* **71**, 1131-1140.

Chen, W. , Carbone, F. R., and McCluskey, J. (1993). Electroporation and commercial liposomes efficiently deliver soluble protein into the MHC class I presentation pathway. Priming in vitro and in vivo for class I-restricted recognition of soluble antigen. *J. Immunol. Methods* **160**, 49-57.

Collins, D. S., Findlay, K. , and Harding, C. V.(1992). Processing of exogenous liposome-encapsulated antigens in vivo generates class I MHC-restricted T cell responses. *J. Immunol.* **148**, 3336-3341.

Czerny, C. P. and Mahnel, H. (1990). Structural and functional analysis of orthopoxvirus epitopes with neutralizing monoclonal antibodies. *J. Gen. Virol.* **71**, 2341-2352.

Germain, R. N.(1991). Antigen presentation. The second class story [news; comment]. *Nature* **353**, 605-607.

Germain, R. N. and Hendrix, L. R.(1991). MHC class II structure, occupancy and surface expression determined by post-endoplasmic reticulum antigen binding. *Nature* **353**, 134-139.

Gheysen, D. , Jacobs, E. , de Foresta, F. , Thiriart, C. , Francotte, M. , Thines, D. , and De Wilde, M. (1989). Assembly and release of HIV-1 precursor Pr55gag virus-like particles from recombinant baculovirus-infected insect cells. *Cell* **59**, 103-112.

Gottlinger, H. G., Sodroski, J. G., and Haseltine, W. A.(1989). Role of capsid precursor processing and myristoylation in morphogenesis and infectivity of human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. U. S. A.* **86**, 5781-5785.

Huang, L. , Reddy, R. , Nair, S. K., Zhou, F. , and Rouse, B. T.(1992). Liposomal delivery of soluble protein antigens for class I MHC-mediated antigen presentation. *Res. Immunol.* **143**, 192-196.

Inazu, M. , Tsuha, O. , Kirisawa, R. , Kawakami, Y. , and Iwai, H. (1993). Equid herpesvirus 1 infection in mice. *J. Vet. Med. Sci.* **55**, 119-121.

Karacostas, V. , Wolffe, E. J., Nagashima, K. , Gonda, M. A., and Moss, B. (1993). Overexpression of the HIV-1 gag-pol polyprotein results in intracellular activation of HIV-1 protease and inhibition of assembly and budding of virus-like particles. *Virology* **193**, 661-671.

Krausslich, H. G., Ochsenbauer, C. , Traenckner, A. M., Mergener, K. , Facke, M. , Gelderblom, H. R., and Bosch, V. (1993). Analysis of protein expression and virus-like particle formation in mammalian cell lines stably expressing HIV-1 gag and env gene products with or without active HIV proteinase. *Virology* **192**, 605-617.

Larsson, M. , Lovgren, K. , and Morein, B. (1993). Immunopotentiation of synthetic oligopeptides by chemical conjugation to iscoms. *J. Immunol. Methods* **162**, 257-260.

Layton, G. T., Harris, S. J., Gearing, A. J., Hill Perkins, M. , Cole, J. S., Griffiths, J. C., Burns, N. R., Kingsman, A. J., and Adams, S. E.(1993). Induction of HIV-specific cytotoxic T lymphocytes in vivo with hybrid HIV-1 V3:Ty-virus-like particles. *J. Immunol.* **151**, 1097-1107.

Lopes, L. M. and Chain, B. M.(1992). Liposome-mediated delivery stimulates a class I-restricted cytotoxic T cell response to soluble antigen. *Eur. J. Immunol.* **22**, 287-290.

Martin, S. J., Vyakarnam, A. , Cheingsong Popov, R. , Callow, D. , Jones, K. L., Senior, J. M., Adams, S. E., Kingsman, A. J., Matear, P. , Gotch, F. M., and et al, (1993). Immunization of human HIV-seronegative volunteers with recombinant p17/p24:Ty virus-like particles elicits HIV-1 p24-specific cellular and humoral immune responses. *AIDS* **7**, 1315-1323.

Nair, S. , Zhou, F. , Reddy, R. , Huang, L. , and Rouse, B. T.(1992). Soluble proteins delivered to dendritic cells via pH-sensitive liposomes induce primary cytotoxic T lymphocyte responses in vitro. *J. Exp. Med.* **175**, 609-612.

Nair, S. , Zhou, X. , Huang, L. , and Rouse, B. T.(1992). Class I restricted CTL recognition of a soluble protein delivered by liposomes containing lipophilic polylysines. *J. Immunol. Methods* **152**, 237-243.

Newman, M. J., Wu, J. Y., Gardner, B. H., Munroe, K. J., Leombruno, D. , Recchia, J. , Kensil, C. R., and Coughlin, R. T.(1992). Saponin adjuvant induction of ovalbumin-specific CD8+ cytotoxic T lymphocyte responses. *J. Immunol.* **148**, 2357-2362.

Nixon, D. F., Huet, S. , Rothbard, J. , Kieny, M. P., Delchambre, M. , Thiriart, C. , Rizza, C. R., Gotch, F. M., and McMichael, A. J.(1990). An HIV-1 and HIV-2 cross-reactive cytotoxic T-cell epitope. *AIDS* **4**, 841-845.

Nixon, D. F., Townsend, A. R., Elvin, J. G., Rizza, C. R., Gallwey, J. , and McMichael, A. J.(1988). HIV-1 gag-specific cytotoxic T lymphocytes defined with recombinant vaccinia virus and synthetic peptides. *Nature* **336**, 484-487.

Osterrieder, N. , Wagner, R. , Pfeffer, M. , and Kaaden, O. R.(1994). Expression of equine herpesvirus type 1 glycoprotein gp14 in Escherichia coli and in insect cells: a comparative study on protein processing and humoral immune responses. *J. Gen. Virol.* **75**, 2041-2046.

Papsidero, L. D., Sheu, M. , and Ruscetti, F. W.(1989). Human immunodeficiency virus type 1-neutralizing monoclonal antibodies which react with p17 core protein: characterization and epitope mapping. *J. Virol.* **63**, 267-272.

Phillips, R. E. and McMichael, A. J.(1993). How does the HIV escape cytotoxic T cell immunity?. *Chem. Immunol.* **56**, 150-164.

Raychaudhuri, S. , Tonks, M. , Carbone, F. , Ryskamp, T. , Morrow, W. J., and Hanna, N. (1992). Induction of antigen-specific class I-restricted cytotoxic T cells by soluble proteins in vivo. *Proc. Natl. Acad. Sci. U. S. A.* **89**, 8308-8312.

Reddy, R. , Nair, S. , Brynestad, K. , and Rouse, B. T.(1992). Liposomes as antigen delivery systems in viral immunity. *Semin. Immunol.* **4**, 91-96.

Sambrook, I., Fritsch, E. F. and Maniatis, T. (1989) Molecular cloning; a laboratory manual. Cold Spring Harbor Press

Smith G. E., Ericson B. L., Moschera J., Lahm H. W., Chizzonite R., Summers M. D. (1985) Modification and secretion of of human interleukin 2 produced in insect cells by a baculovirus expression vector. *Proc. Natl. Acad. Sci. USA.* **82**:8404-8408

Steimer, K. S. and Haigwood, N. L.(1991). Importance of conformation on the neutralizing antibody response to HIV-1 gp120. *Biotechnol. Ther.* **2**, 63-89.

Takahashi, H. , Cohen, J. , Hosmalin, A. , Cease, K. B., Houghten, R. , Cornette, J. L., DeLisi, C. , Moss, B. , Germain, R. N., and Berzofsky, J. A.(1988). An immunodominant epitope of the human immunodeficiency virus envelope glycoprotein gp160 recognized by class I major histocompatibility complex molecule-restricted murine cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. U. S. A.* **85**, 3105-3109.

Takahashi, H. , Takeshita, T. , Morein, B. , Putney, S. , Germain, R. N., and Berzofsky, J. A.(1990). Induction of CD8+ cytotoxic T cells by immunization with purified HIV-1 envelope protein in ISCOMs [see comments]. *Nature* **344**, 873-875.

Townsend, A. and Bodmer, H. (1989). Antigen recognition by class I-restricted T lymphocytes. *Annu. Rev. Immunol.* **7**, 601-624.

van Binnendijk, R. S., van Baalen, C. A., Poelen, M. C., de Vries, P. , Boes, J. , Cerundolo, V. , Osterhaus, A. D., and Uytdehaag, F. G.(1992). Measles virus transmembrane fusion protein synthesized de novo or presented in immunostimulating complexes is endogenously processed for HLA class I- and class II-restricted cytotoxic T cell recognition. *J. Exp. Med.* **176**, 119-128.

Wagner, R. , Boltz, T. , Deml, L. , Modrow, S. , and Wolf, H. (1993). Induction of cytolytic T lymphocytes directed towards the V3 loop of the human immunodeficiency virus type 1 external glycoprotein gp120 by p55gag/V3 chimeric vaccinia viruses. *J. Gen. Virol.* **74**, 1261-1269.

Wagner, R. , Deml, L. , Fliessbach, H. , Wanner, G. , and Wolf, H. (1994). Assembly and extracellular release of chimeric HIV-1 Pr55gag retrovirus-like particles. *Virology* **200**, 162-175.

Wagner, R. , Fliessbach, H. , Modrow, S. , von Brunn, A. , Wolf, H. , Boelz, T. , and Gelderblom, H. (1991). *Modern approaches to new vaccines including prevention of AIDS. In "Vaccines 91"* (Chanock, R. M., Ginsberg, H. S., Brown, F. , and Lerner, R. A. -., Eds.) Cold Spring Habour Laboratory, Cold Spring Habour, NY. . 109-114.

Wagner, R. , Fliessbach, H. , Wanner, G. , Motz, M. , Niedrig, M. , Deby, G. , von Brunn, A. , and Wolf, H. (1992). Studies on processing, particle formation, and

immunogenicity of the HIV-1 gag gene product: a possible component of a HIV vaccine. *Arch. Virol.* **127**, 117-137.

Wagner, R. , Modrow, S. , Boltz, T. , Fliessbach, H. , Niedrig, M. , von Brunn, A. , and Wolf, H. (1992). Immunological reactivity of a human immunodeficiency virus type I derived peptide representing a consensus sequence of the GP120 major neutralizing region V3. *Arch. Virol.* **127**, 139-152.

Yewdell, J. W. and Bennink, J. R.(1992). Cell biology of antigen processing and presentation to major histocompatibility complex class I molecule-restricted T lymphocytes. *Adv. Immunol.* **52**, 1-123.

Zhou, F. , Rouse, B. T., and Huang, L. (1992). Induction of cytotoxic T lymphocytes in vivo with protein antigen entrapped in membranous vehicles. *J. Immunol.* **149**, 1599-1604.

CLAIMS

1. An antigen presentation system comprising:
   (a) a DNA sequence encoding a protein capable of self assembly into proteinaceous particles without a lipid membrane, preferably a retroviral group specific antigen (gag) capable of forming a particulate structure when expressed in a host cell and of being delivered into the extracellular medium; and
   (b) a DNA sequence encoding
      (ba) a signal peptide; and operatively linked thereto
      (bb) an extracellular domain of a polypeptide;
      (bc) a transmembrane region; and
      (bd) a cytoplasmic region which does not comprise the amino acid sequence that mediates a specific interaction with the matrix protein:

   wherein said DNA sequences (a) and (b) are together expressible in a suitable host cell.

2. The antigen presentation system according to claim 1, wherein the signal sequence and/or the transmembrane region and/or the cytoplasmic region are heterologous with regard to the extracellular domain.

3. The antigen presentation system according to claim 1, wherein the signal sequence and/or the transmembrane region and/or the cytoplasmic region are autologous with regard to the extracellular domain.

4. The antigen presentation system according to any one of claims 1 to 3, wherein interactive sites of the cytoplasmic amino acid sequence have been deleted.

5. The antigen presentation system according to any one of claims 1 to 3, wherein said amino acid sequence in said cytoplasmic domain has been mutagenized so as to not mediate the specific interaction with the matrix protein.

6. The antigen presentation system according to any one of claims 1 to 5, wherein said extracellular domain of a polypeptide is derived from a pathogenic agent.

7. The antigen presentation system according to claim 6, wherein said pathogenic agent is a virus, a bacterium, a prion or a neoplastic cell.

8. The antigen presentation system according to claim 7, wherein said virus is HIV-1, HIV-2, HTLV-1, HTLV-2, SIV or FIV, Epstein-Barr virus or a herpes virus such as equine herpes virus.

9. The antigen presentation system according to any one of claims 6 to 8, wherein said extracellular domain of said polypeptide is an authentic extracellular domain.

10. The antigen presentation system according to any one of claims 6 to 8, wherein said extracellular domain of said polypeptide is a non-authentic extracellular domain and preferably a chimeric extracellular domain.

11. The antigen presentation system according to any one of claims 7 to 10, wherein said extracellular domain is derived from an env protein.

12. The antigen presentation system according to any one of claims 1 to 11, wherein said gag is derived from HTLV-1, HTLV-2, HIV-1, HIV-2, SIV or FIV.

13. The antigen presentation system according to claim 12, wherein said gag is $pr55^{gag}$ of HIV-1.

14. The antigen presentation system according to any one of claims 1 to 13, wherein said signal peptide is derived from an interleukin-3 signal peptide,.

15. The antigen presentation system according to any one of claims 1 to 14, wherein said transmembrane region is derived from the EBV gp220/350 transmembrane anchor region or from a herpes virus, optionally connected via a glycine/serine linker with said extracellular domain.

16. The antigen presentation system according to any one of claims 1 to 15, wherein said DNA sequence (a) and (b) are contained in the same expression vector.

17. The antigen presentation system according to any one of claims 1 to 15, wherein said DNA sequences (a) and (b) are contained in different expression vectors.

18. The antigen presentation system according to claim 16 or 17, wherein said vector(s) is/are (a) baculovirus derived vector(s) or semliki forest virus based vector(s).

19. The antigen presentation system according to any one of claims 1 to 18, which is expressible in eukaryotic cells, preferably mammalian cells or insect cells, preferably Drosophila Schneider cells.

20. A host cell transfected with the antigen presentation system according to any one of claims 1 to 19.

21. The host cell according to claim 20, which is a mammalian or an insect cell, preferably a Drosophila Schneider cell.

22. An antigenic polypeptide encoded by the antigen presentation system according to any one of claims 1 to 19 or produced by the host cell according to claim 20 or 21.

23. A pharmaceutical composition or vaccine comprising the antigen presentation system according to any one of claims 1 to 19, the host cell according to claim 20 or 21 and/or the polypeptide according to claim 22.

24. A diagnostic composition comprising the polypeptide of claim 22.

25. A method of producing the polypeptide of claim 22, comprising culturing the host cell according to claim 20 or 21 in a suitable culture medium and collecting immature virus-like particles carrying said polypeptides produced by said host cells from the medium.

26. A method for eliciting an immune response specific to the route of antigen administration which is topical, preferably via mucosal exposure or invasive, preferably via injection or scarification.

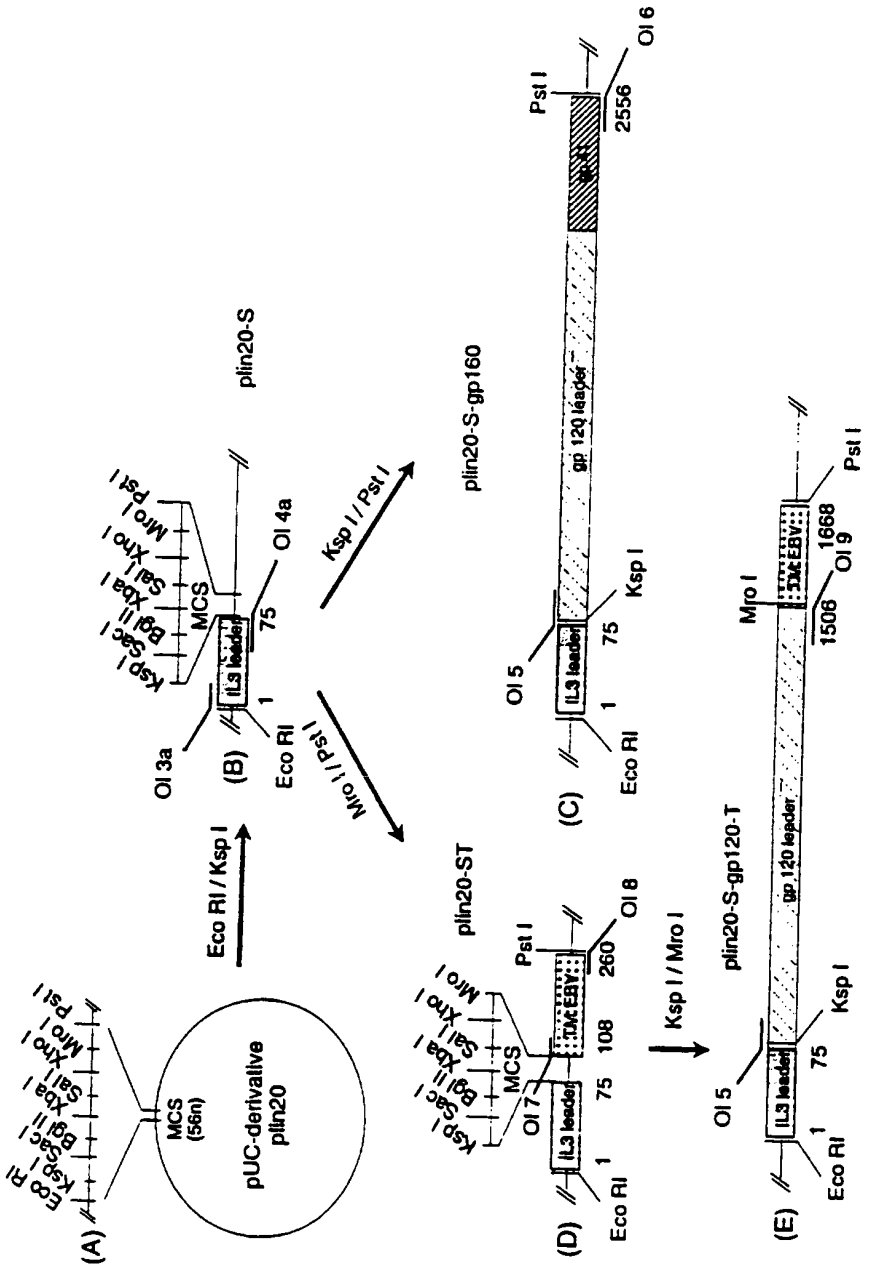

pUC-derivative plin20
MCS (56n)
Eco RI / Ksp I
plin20-S
plin20-S-gp160
plin20-ST
plin20-S-gp120-T
Ksp I / Pst I
Mro I / Pst I
Ksp I / Mro I
IL3 leader
gp 120 leader
TMCEBV
Eco RI
Ksp I
Pst I
Mro I
Sal I
Xho I
Xba I
Bgl II
Sac I
(A)
(B)
(C)
(D)
(E)
Ol 3a
Ol 4a
Ol 5
Ol 6
Ol 7
Ol 8
Ol 9
75
1
108
260
2556
1506
1668

Figure 1

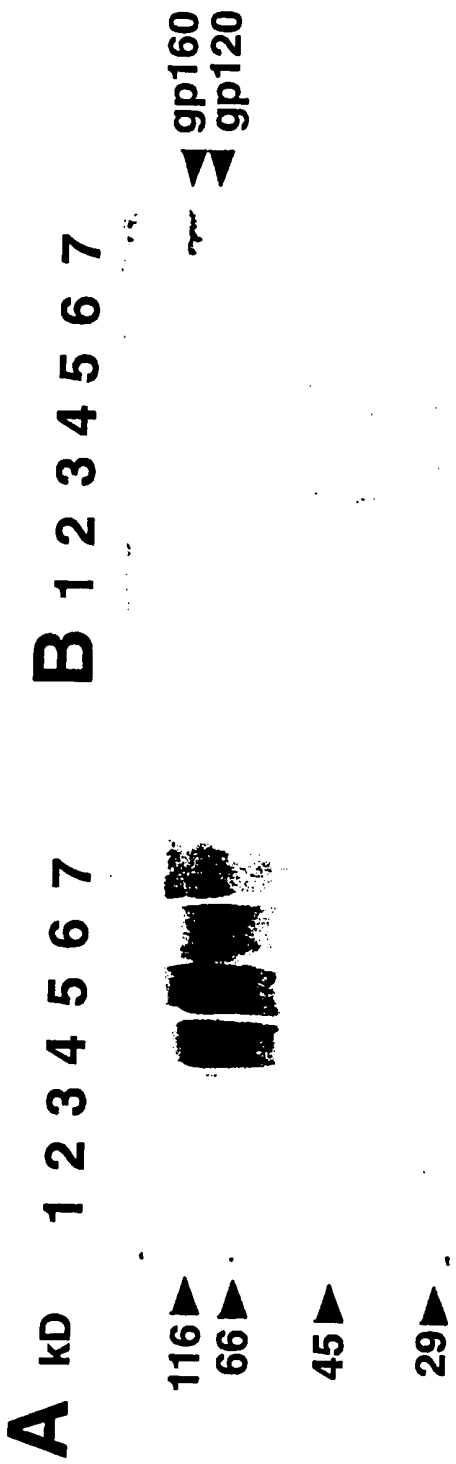


Figure 2

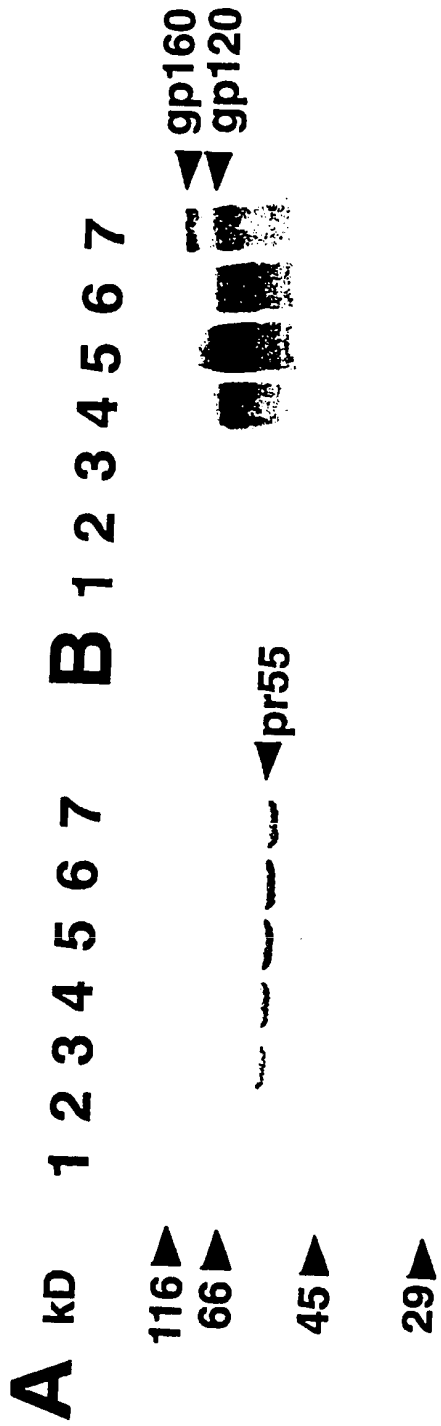


Figure 3

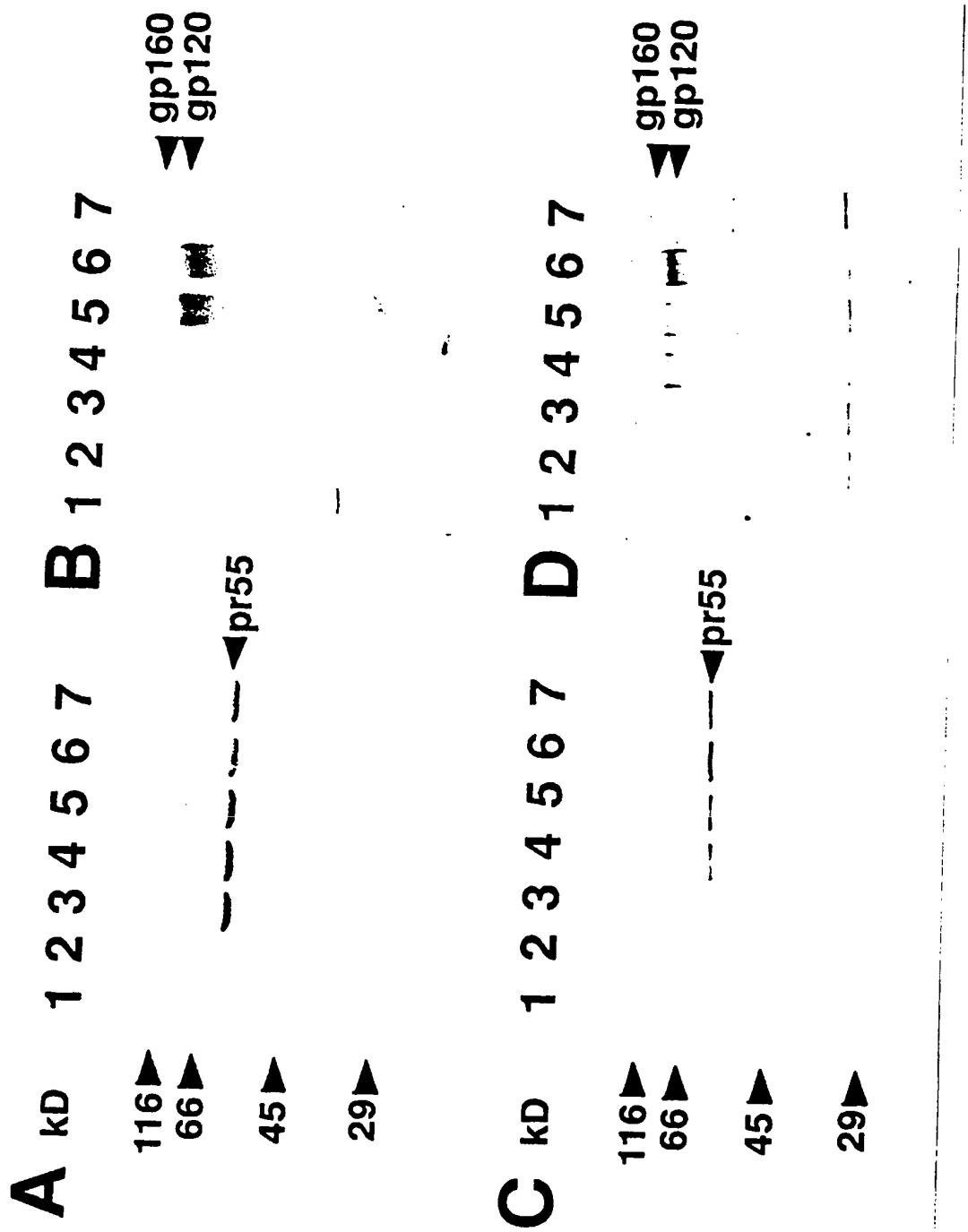

A
kD
1 2 3 4 5 6 7
116
66
45
29
pr55
gp160
gp120
B
1 2 3 4 5 6 7
C
kD
1 2 3 4 5 6 7
116
66
45
29
pr55
gp160
gp120
D
1 2 3 4 5 6 7

Figure 4

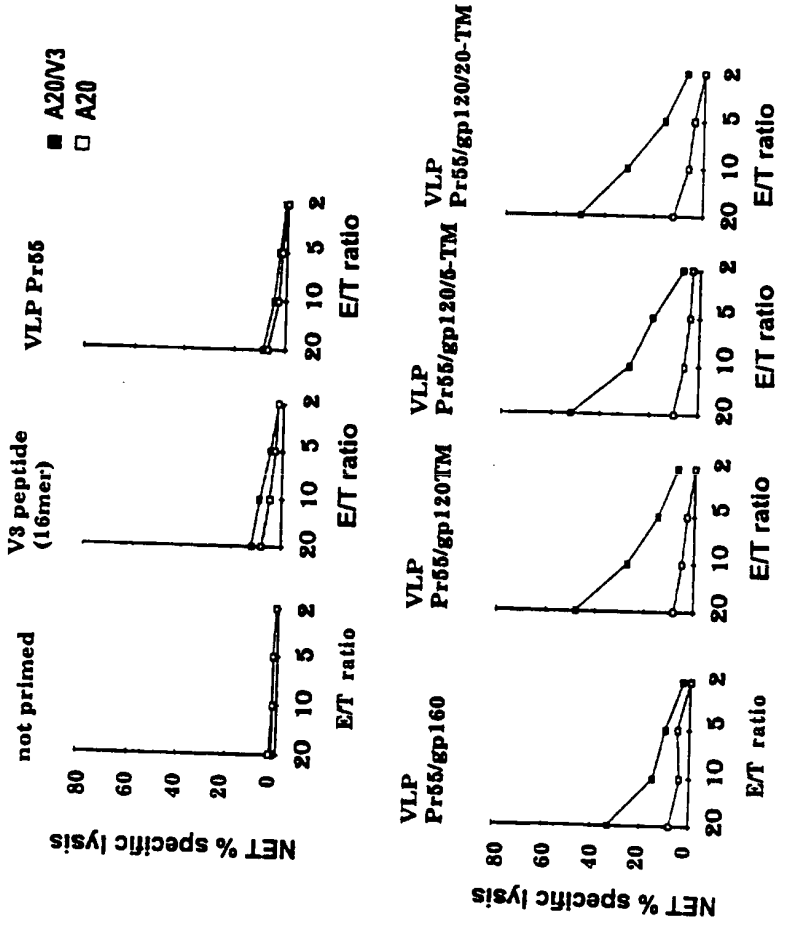

■ A20/V3
□ A20
not primed
V3 peptide (16mer)
VLP Pr55
VLP Pr55/gp160
VLP Pr55/gp120TM
VLP Pr55/gp120/5-TM
VLP Pr55/gp120/20-TM
NET % specific lysis
80
60
40
20
0
E/T ratio
20 10 5 2

Figure 5

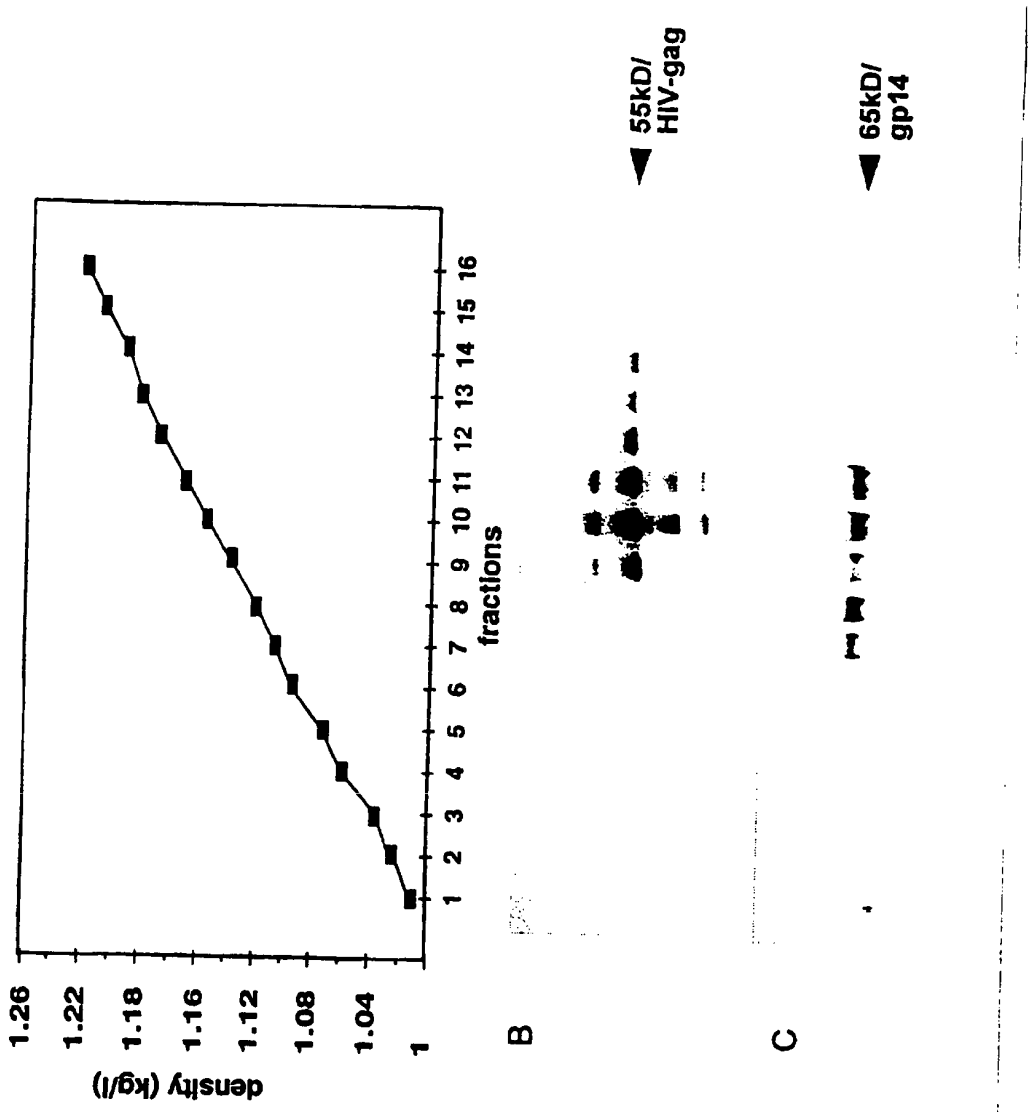
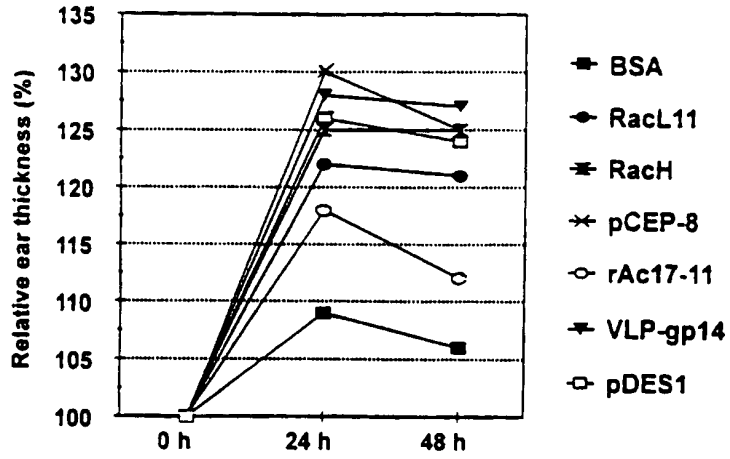

density (kg/l)
1.26
1.22
1.18
1.16
1.12
1.08
1.04
1
fractions
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
B
55kD/
HIV-gag
C
65kD/
gp14

Figure 6

Figure 7

A.


Relative ear thickness (%)
135
130
125
120
115
110
105
100
0 h
24 h
48 h
BSA
RacL11
RacH
pCEP-8
rAc17-11
VLP-gp14
pDES1

B.

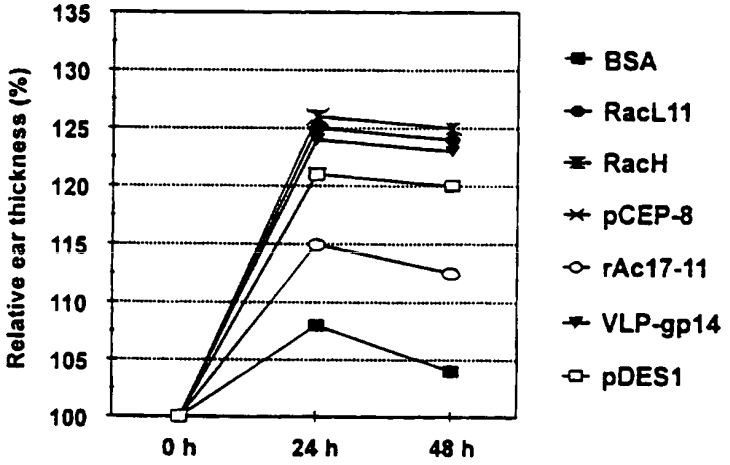
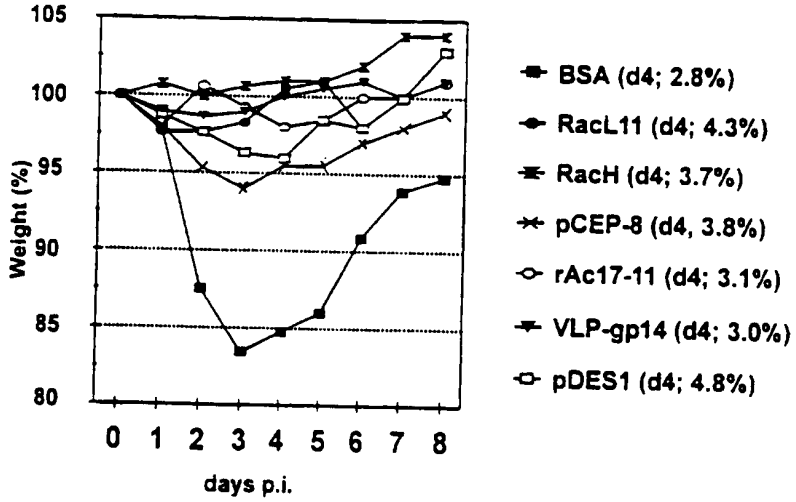

Relative ear thickness (%)
135
130
125
120
115
110
105
100
0 h
24 h
48 h
BSA
RacL11
RacH
pCEP-8
rAc17-11
VLP-gp14
pDES1

Figure 8

A.


105
100
95
90
85
80
Weight (%)
0 1 2 3 4 5 6 7 8
days p.i.
BSA (d4; 2.8%)
RacL11 (d4; 4.3%)
RacH (d4; 3.7%)
pCEP-8 (d4, 3.8%)
rAc17-11 (d4; 3.1%)
VLP-gp14 (d4; 3.0%)
pDES1 (d4; 4.8%)

B.

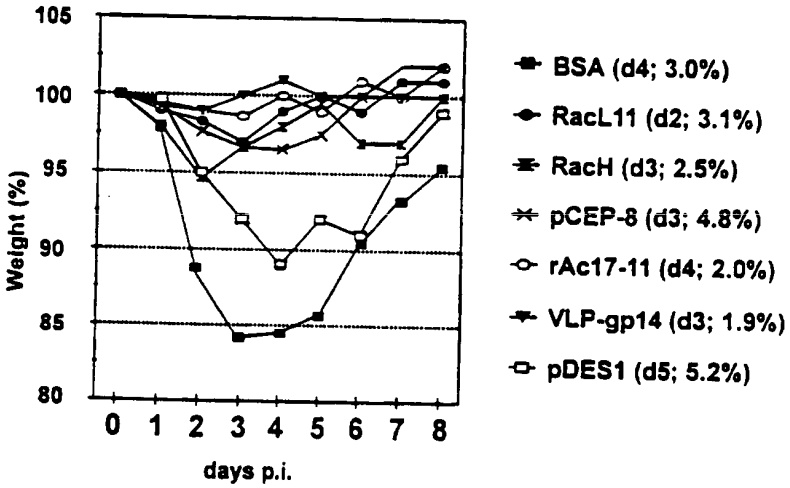

105
100
95
90
85
80
Weight (%)
0 1 2 3 4 5 6 7 8
days p.i.
BSA (d4; 3.0%)
RacL11 (d2; 3.1%)
RacH (d3; 2.5%)
pCEP-8 (d3; 4.8%)
rAc17-11 (d4; 2.0%)
VLP-gp14 (d3; 1.9%)
pDES1 (d5; 5.2%)

Figure 9

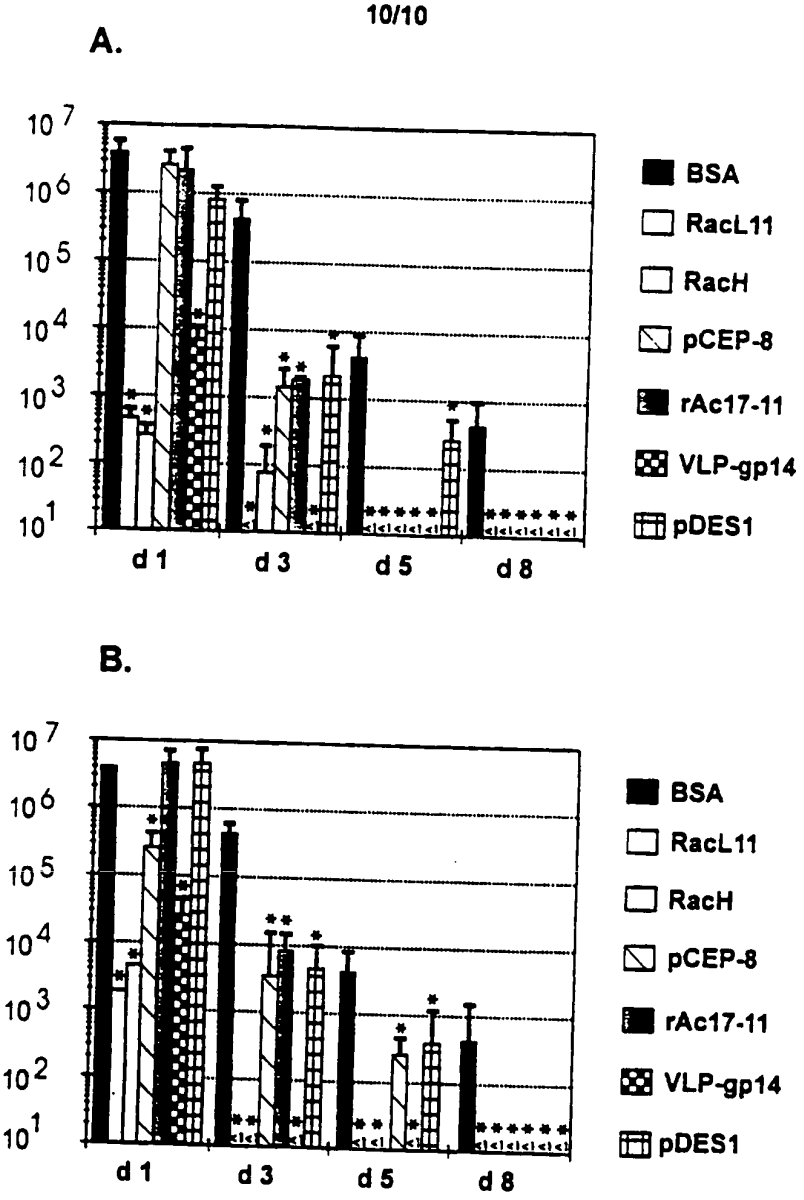

A.
10 7
10 6
10 5
10 4
10 3
10 2
10 1
d 1
d 3
d 5
d 8
BSA
RacL11
RacH
pCEP-8
rAc17-11
VLP-gp14
pDES1
B.
10 7
10 6
10 5
10 4
10 3
10 2
10 1
d 1
d 3
d 5
d 8
BSA
RacL11
RacH
pCEP-8
rAc17-11
VLP-gp14
pDES1

Figure 10